IN VITRO STUDIES ON TISSUE, CELL AND PROTOPLAST CULTURE OF THE GENUS *PAULOWNIA*

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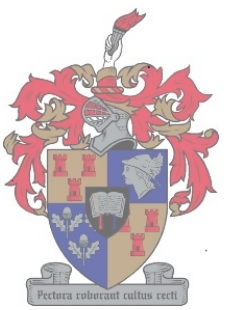

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Declaration

I the undersigned hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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OPSOMMING

IN VITRO STUDIES VAN WEEFEEL-, SEL- EN PROTOPLAST KULTURE VAN DIE GENUS *PAULOWNIA*

Die doel van hierdie studie was om *in vitro* sisteme te ontwikkel vir organogenese en embriogenese in weefsel-en kalluskulture, selsuspensie en selkultuur sowel as protoplast isolasie. Drie spesies van die genus *Paulownia* is geselekteer nl. *P.fortunei*, *P.kawakamii* en *P.tometosa*. *P.taiwaniana* is ook ingesluit vir vergelykende doeleindes aangesien baie *in vitro* studies op hierdie spesies gedoen is.

Die ontkieming van vier *Paulownia* spesies in verskillende plantingsmedia is ondersoek om sodoende *in vitro* saailinge vir eksperimentele gebruik te verkry. Volwasse en onvolwasse sade van al vier *Paulownia* spesies is geweek in 'n 3.5 % natriumhipochloriet oplossing om ontkieming te stimuleer. Hoër ontkiemingspersentasies is verkry nadat die sade eers met 70 % alkohol vir 1 min behandel is voordat dit geweek is in natriumhipochloriet. MS medium het ontkieming van die volwasse sade geinhibeer, maar ontkieming van onvolwasse sade is nie tot dieselfde mate daardeur beïnvloed nie. Die invloed van die afsonderlike komponente van MS medium op die ontkieming van *P.tomentosa* is ontleed en daar is gevind dat sukrose, totale soute en sommige makrovoedingstowwe die grootste inhiberende invloed gehad het. *Paulownia* sade moet dus ontkiem word op water wat gestol is met 0.6 % agar voordat dit oorgeplaas word na MS medium vir *in vitro* groei.

Organogenese in eksplante van vier *Paulownia* spesies is ondersoek. Saadlob, hipokotiel, lootpunt, node, internode en blaar eksplante is getoets. Eksplante is gekweek op MS medium wat aangevul is met verskillende kombinasies van NAS en

BA. Alle eksplant tipes van *P.tomentosa* en *P.taiwaniana* sowel as saadlob en hipokotiel eksplante van *P.fortunei* en *P.kawakamii* het adventiewe knoppe binne die konsentrasie gebied van 0.5 mg l^{-1} NAS en 5 mg l^{-1} BA, gevorm. Hipokotiel eksplante het die meeste adventiewe knoppe gevorm terwyl blaar eksplante die minste geproduseer het. Adventiewe knop- en wortelvorming op die ander vier eksplante van *P.fortunei* en *P.kawakamii* het met 'n wye reeks NAS en BA kombinasies voorgekom. Biolab agar het in teenstelling met Difco agar adventiewe knopvorming by internodale eksplante van *P.tomentosa* geïnhibeer. By al vier spesies het Biolab agar aanleiding gegee tot meer kallusgroei as Difco agar. *P.fortunei* en *P.tomentosa* het makliker kallus gevorm as die ander twee spesies.

Lote wat *in vitro* afgekweek is op gelrite, het aanleiding gegee tot uitgebreide verglasing op 'n media sonder hormone en wat gestol is met gelrite. Verskillende konsentrasies agar, kobalt, inositol, MS makrosoute, groeireguleerder en verskillende ligintensiteite het nie die verglasing probleem uitgeskakel nie. 'n Toename in ligintensiteit het adventiewe knopvorming verhoog. *P.taiwaniana* het minder verglaasde lote gevorm as *P.fortunei* en *P.kawakamii*. Gelrite het meer adventiewe knoppe laat vorm, maar dit het ook meer verglasing as agar veroorsaak. Die morfologiese eienskappe en groei van beide normale en verglaasde lote is ondersoek nadat hulle afgekweek is op verskillende gelagente en konsentrasies. Verglaasde lote van *P.fortunei* en *P.tomentosa* het meer kallus geproduseer op die snyoppervlaktes, terwyl die ander twee spesies meer oksilêre lote gevorm het. Verglaasde lote het 'n hoër groeitempo getoon as normale lote, maar daar was geen verskil tussen die wortelvormingvermoëns van die twee tipes lote nie. Lootgroei is geinhibeer deur 'n toename in gelagent konsentrasie, behalwe in die geval van *P.kawakamii* wat stadiger gegroei het op al die konsentrasies gelagente getoets. Verglaasde lote wat gekweek is

op 0.8 % of hoër Difco agar se waterinhoud het gedaal en hulle het herstel na normale lote. Dit het egter nie gebeur op gelrite of mengsels van gelrite en agar nie.

Die induksie van somatiese embriogenese in vier *Paulownia* spesies is ondersoek. Saadknoppe van *P.fortunei*, in die globulêre proembrio stadium, is gekweek op Radojevic (1979), MSG (Amerson *et al.*, 1988) en DCR (Gupta & Durzan, 1986) media wat aangevul is met ouksiene en sitokiniene. Hierdie saadknoppe het ontwikkel tot volwasse embrios of kallusvorming het plaasgevind. Geen verdere ontwikkeling het plaasgevind nadat die embrio en kallus afgekweek is nie. Volwasse en onvolwasse sade gekweek op verskeie media het lae ontkieming getoon. Ontkiemende embrios is gekweek op Radojevic medium of MGM medium (Muralidharan *et al.*, 1989) wat aangevul is met 2.5 % sukrose en picloram, 2,4-D of NAS gekombineer met kinetien of BA. Geen embriogeniese weefsel het in die lig of donker gevorm nie. Embriogenese in *P.tomentosa* kon nie volgens die prosedure beskryf deur Radojevic (1979) geinduseer word nie.

Die gedrag van kallusweefsel in MS media wat NAS of 2,4-D plus BA of kinetien bevat, is ondersoek. Hierdie kallusweefsel is verkry vanaf verskillende eksplant tipes van vier *Paulownia* spesies, wat gekweek is op MS medium aangevul met verskeie kombinasies van NAS + BA of 2,4-D + kinetien. Organogenetiese kallus met 'n kompakte struktuur en' n groenerige - wit kleur, is geinduseer met lae konsentrasies NAS (< 0.5 mg l^{-1}) of in kombinasies van NAS en BA sowel as met lae vlakke van 2,4-D plus kinetien. Krummelrige kallus met 'n wit of gryserïge kleur het in hoë vlakke van 2,4-D (0.1 mg l^{-1}) plus kinetien ontwikkel. Afgekweekte organogenetiese kallusse wat verkry is in medium (0.5 mg l^{-1} NAS en 5 mg l^{-1} BA) wat adventiewe knopvorming geïnduseer het, kon nie behou word nadat dit afgekweek is nie. Organogenetiese kallusse wat verkry is vanaf 'n medium wat 0.1 mg l^{-1} 2,4-D en 1 mg

l^{-1} kinetien bevat het, en wat veroorsaak het dat min adventiewe knoppe gevorm het, is geïnduseer om 'n groot aantal adventiewe knoppe te laat ontwikkel nadat dit afgekweek is op 0.01 mg l^{-1} NAS en 10 mg l^{-1} BA in die geval van *P.taiwaniana*, maar nie vir *P.kawakamii* nei. Die meeste kallusse, organogeneties of krummelrig, het bruin verkleur nadat dit afgekweek is. Behandelings met antioksidante het nie die verbruining verminder nie. Verbruining was minder opvallend waar adventiewe knoppe teenwoordig was.

Selsuspensies van *Paulownia tomentosa* is verkry vanaf krummelrige kallus wat vanaf lootpunt eksplante gekweek is op MS medium wat 2.5 mg l^{-1} NAS en BA bevat het. Selsuspensies van *P.tomentosa* en *P.taiwaniana* is ook verkry sonder 'n kallus interfase, vanaf saailinglote in kultuur. MS medium aangevul met 1 mg l^{-1} 2,4-D en 0.1 mg l^{-1} kinetien is gebruik. Kulture is in die donker gehou op 'n roterende skudapparaat teen 100 opm. Seldigthede van 4.5×10^5 is behaal deur gebruik te maak van 200 mg eksplantweefsel. Die meeste selle (75 - 95 %) was lewensvatbaar en selsuspensies van *P.tomentosa* en *P.taiwaniana* het 'n hoë groeitempo gehad. Die kulture is vir langer as 1 jaar behou deur hulle gereeld af te kweek. Dieselfde medium is gebruik om *P.tomentosa* selle af te kweek, maar om *P.taiwaniana* suksesvol af te kweek is die konsentrasie 2,4-D en kinetien verminder. Verder was NAS en BA ook noodsaaklik. Hierdie metode om die kweking van selsuspensie tot stand te bring is effektief en 'n besparing van tyd in vergelyking met die algemeen aanvaarde metode wat van kallusweefsel gebruik maak. Die produksie van geskikte krummelrige kallus op agar vir die kweek van suspensies word dus uitgeskakel. Vir beide spesies is kallusvorming vanaf suspensie selle verkry deur selle te plaat of om suspensieselle oor te plaas na 'n vloeibare MS medium wat NAS en BA bevat. Organogeneses vanaf kallusse is egter nie behaal nie.

Die isolasie van protoplaste vanaf *in vitro* eksplante van *P.tomentosa*, *P.kawakamii* en *P.taiwaniana* sowel as suspensieselle van *P.taiwaniana* is ondersoek. Blare van *P.tomentosa* het meer vry mesofilselle voortgebring nadat dit met Seravac pektinase behandel is as enige ander handelsnaam pektinase. Hoë opbrengste van protoplaste is verkry nadat eksplante in 'n ensiemoplossing van 0.6 % pektinase, 0.6 % hemisellulase en 2 % Onozuka R10 sellulase geïnkubeer is. *P.taiwaniana* suspensie selle wat behandel is met 2 % Seravac sellulase het hoë protoplast opbrengste gelewer. Baie *P.taiwaniana* suspensieselle het nie volkome in 'n mannitol oplossing geplasmoliseer nie vanweë die sterk binding wat tussen die membraan en selwand bestaan. Selle is beskadig in konsentrasies hoër as 0.6 M mannitol en wanneer die inkubasieperiode langer as 5 ure geduur het. Protoplaste vanaf *P.tomentosa* mesofilselle het nie daarin geslaag om te verdeel nie. Die aanvangsfase van verdeling in protoplaste van P.*taiwaniana* suspensie selle was sigbaar maar verdeling is nie voltooi nie.

ABSTRACT

The aim of this study was to establish an *in vitro* system of *Paulownia* species for organogenesis and embryogenesis in tissue and callus cultures, cell suspension and cell culture, protoplast isolation and culture. Three species viz. *P.fortunei*, *P.kawakamii*, *P.tomentosa* which represent three sections of *Paulownia* genus were selected. *P.taiwaniana* was also included for comparative purposes since many *in vitro* studies have been done on this species.

To obtain *in vitro* seedlings for experimental usage seed germination of four *Paulownia* species sown in different planting media was studied. Germination of both mature and immature seeds for all *Paulownia* species was stimulated by soaking seeds in a 3.5 % sodium hypochlorite solution. Pretreatment of the seeds with 70 % ethanol for 1 min before soaking in sodium hypochlorite caused higher germination percentages than sodium hypochlorite treatment alone. MS medium inhibited germination of mature seeds, but germination of immature seeds was not affected to the same extent. Analyses of the effect of individual components of MS medium on the germination of *P.tomentosa* revealed that sucrose, the total salts and some macronutrients were the major inhibitory factors. *Paulownia* seeds should therefore be germinated on water solidified with 0.6 % agar before transfer to MS medium for *in vitro* growth.

Organogenesis in explants of four *Paulownia* species was studied. Cotyledon, hypocotyl, shoot tip, nodal, internodal and leaf explants were tested. Explants were cultured on MS medium supplemented with different combinations of NAA and BA. All explant types of *P.tomentosa* and *P.taiwaniana* and cotyledon and hypocotyl explants of *P.fortunei* and *P.kawakamii* formed adventitious buds within the

concentration range of 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA. Hypocotyl explants produced more adventitious buds than the other explants, while leaf explants produced the least. Adventitious bud and root formation on the other four explants for *P.fortunei* and *P.kawakamii* occurred over a wide range of NAA + BA combinations. Adventitious bud formation of internodal explants of *P.tomentosa* was inhibited by Biolab agar but not by Difco agar. Biolab agar induced more callus growth than Difco agar for all four species. *P.fortunei* and *P.tomentosa* formed callus more readily than the other two species.

Extensive vitrification occurred in *in vitro* shoot subcultures on MS medium solidified with gelrite and adventitious buds formed from explants cultured on medium containing hormones. To reduce vitrification of adventitious buds, internodal explants of four *Paulownia* species were cultured on shoot formation media with different concentrations of agar, cobalt, inositol, MS macrosalts, growth regulators and different of light intensities. None of these treatments gave both a high adventitious bud formation and a low incidence of vitrification. Increasing light intensity increased adventitious bud formation. *P.taiwaniana* formed fewer vitrified shoots than *P.fortunei* and *P.kawakamii*. Gelrite formed more adventitious buds but caused more vitrification than agar. The morphological characteristics and growth of normal and vitrified shoots were studied when subcultured on different gelling agents and concentrations. Vitrified shoots of *P.fortunei* and *P.tomentosa* produced more callus on the cut surfaces of shoots whereas the other two species formed more axillary shoots. Vitrified shoots had a higher growth rate than normal shoots but the ability to form roots did not differ from normal shoots. Increasing the concentration of gelling agents inhibited shoot growth except for *P.kawakamii* which had an inherent slow growth rate on all concentrations of the gelling agents tested. Vitrified shoots decreased water content and reverted to

normal shoots when cultured on 0.8 % or higher Difco agar, but not on gelrite and mixtures of gelrite and agar.

Induction of somatic embryogenesis in four species of *Paulownia* was investigated. Ovules of *P.fortunei* at the globular proembryo stage cultured on Radojevic (1979) , MSG (Amerson *et al.*, 1988) and DCR (Gupta & Durzan, 1986a) media supplemented with auxins and cytokinins developed into mature embryos or formed callus. Neither callus nor embryos developed further when subcultured. Mature and immature seeds cultured on various media germinated in low numbers. Germinating embryos, cultured on Radojevic (1979) medium or MGM medium (Muralidharan *et al.*, 1989) supplemented with 2, 5 % sucrose and picloram, 2,4-D or NAA combined with kinetin or BA in light or dark, failed to form embryogenic tissue. Embryogenesis could not be induced in *P.tomentosa* by the procedure described by Radojevic (1979).

The behavior of callus tissue derived from different explant types of four *Paulownia* species cultured on MS medium plus various combinations of NAA + BA or 2,4-D + kinetin was studied in MS media containing NAA or 2,4-D plus BA or kinetin. Organogenetic callus compact in structure and greenish white in colour were induced in low levels of NAA (< 0.5 mg l^{-1}) or in combinations of NAA and BA or in low levels of 2,4-D plus kinetin. Friable callus white or greyish in colour developed in high levels of 2,4-D (0.1 mg l^{-1}) plus kinetin. Subcultures of organogenetic calli derived from a medium (0.5 mg l^{-1} NAA and 5 mg l^{-1} BA) which induced adventitious bud formation could not be maintained when subcultured. Organogenetic callus derived from a medium containing 0.1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin which caused few adventitious buds to form were induced to form many adventitious buds when subcultured on 0.01 mg l^{-1} NAA and 10 mg l^{-1} BA in the case of *P.taiwaniana* but not for *P.kawakamii*. Most callus whether organogenetic or friable turned brown when

subcultured. Treatments with antioxidant did not reduce browning. Browning of cultures with adventitious buds was less evident than in cultures without adventitious buds.

Cell suspensions of *Paulownia tomentosa* were obtained from friable calli which were obtained by culturing shoot tip explants on MS medium containing 2.5 mg l^{-1} NAA and BA. Cell suspensions of *P.tomentosa* and *P.taiwaniana* were also obtained without callus interphase by culturing seedling shoots. MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin were used. Cultures were kept in the dark on a gyratory shaker at 100 rpm. Cell densities of 4.5 x 10^5 were achieved by using 200 mg explants. Most cells (75 - 95 %) were viable and cell suspensions of *P.tomentosa* and *P.taiwaniana* had a high growth rate. The cultures were maintained for more than one year by regular subculturing. The same medium was used to subculture *P.tomentosa* cells, but for successful subculture of *P.taiwaniana* 2,4-D and kinetin concentration was decreased and NAA and BA was necessary. This method of establishing cell suspension cultures is effective and time-saving compared to the generally accepted method using callus tissue. The production of friable callus on agar suitable for suspension culture is eliminated. Callus formation from suspension cells for both species were achieved by cell plating or transferring suspension cells into liquid MS medium containing NAA and BA. Organogenesis from callus was, however, not achieved.

Isolation of protoplasts from *in vitro* explants of *P.tomentosa*, *P.kawakamii* and *P.taiwaniana* and suspension cells of *P.taiwaniana* were studied. Leaves of *P.tomentosa* treated with Seravac pectinase yielded more free mesophyll cells than other brands of pectinase. High yields of protoplasts were obtained when explants were incubated in an enzyme solution consisting of 0.6 % pectinase, 0.6 %

hemicellulase and 2 % Onozuka R10 cellulase. High yields of protoplasts from suspension cells of *P.taiwaniana* were obtained when cells were treated with 2 % Seravac cellulase. Suspension cells of *P.taiwaniana* exhibited strong membrane-to-cell wall attachment and many cells failed to plasmolyze completely in a mannitol solution. Injury to the cells was evident in concentrations in excess of 0.6 M mannitol and incubation times longer than 5 hours. Protoplast from mesophyll cells of *P.tomentosa* failed to divide while initial stages of division in protoplast from suspension cells of *P.taiwaniana* was evident, but division was not completed.

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CHAPTER 1

REVIEW OF LITERATURE

1. INTRODUCTION

Paulownia has been known and cultivated in China since the book of Erh-ya, the first Chinese encyclopedia on natural and cultural objects, written probably about the third century BC. It was introduced from eastern Asia into Europe in 1830, into America around 1844, and into Australia in 1922 as ornamental trees (Hu, 1959). Countries such as USA, Brazil and South Africa have tested *Paulownia* species for wood production. Thirteen thousand hectares of *Paulownia taiwaniana* plantation were planted in Brazil during 1973-1974 (Rin, 1975; Donald, 1990). The characteristics, economical potential, problems in cultivation, and strategies to improve the genetic potential of the crop by combining conventional breeding with plant tissue and cell culture of *Paulownia* are discussed:

1.1 Taxonomy and distribution

The genus *Paulownia* is the one of few arborescent genera in the family Scrophulariaceae. It is indigenous to the temperate zones of eastern Asia (Hu, 1959; Chinese Academy of Forestry, 1986). Hu (1959) grouped *Paulownia* into 3 sections with 6 species: Paulownia (type species is *P.tomentosa* (Thunb.) Steud.), Fortunei (type species is *P.fortunei* (Seem.) Hemsley.) and Kawakamii (type species is *P.kawakamii* Ito.). A later systematic survey of *Paulownia* by the Chinese Academy of Forestry classified the genus into 9 species (Chinese Academy of Forestry, 1986).

P.taiwaniana has been shown to be a hybrid between *P.fortunei* and *P.kawakamii* (Lin & Wang, 1991).

Paulownia species are attractive, deciduous trees. The leaves are usually sparse and the crown is terete or umbrella-like. Flowers are large and colours range from white to purple and appear in the spring before the leaves. The fruits are loculicidal capsules containing thousands of seeds (Hu, 1959; Chinese Academy of Forestry, 1986).

The natural distribution of *Paulownia* covers the northern area of Liaoning, Beijing, Taiyuan, Yianan and Pinglian counties, extending to the southern areas of Kwantung and Kwangsi counties of mainland China and Taiwan (Fig. 1. 1). Most species with the exception of *P.catalpifolia* and *P.taiwaniana* occur in the middle and lower valleys of the Yangtze River (Chinese Academy of Forestry, 1986).

1.2 Economic potential

The rapid growth rate, drought resistance and tolerance of poor soils adds to the commercial value of *Paulownia* (Howlett, 1975; Burger, 1989). Under normal conditions, a 10-year old *Paulownia* tree can reach a diameter of 30 - 40 cm at breast height with a timber volume of 0.3 - 0.5 m^3 (Chinese Academy of Forestry, 1986). However, under optimum conditions as found in Brazil, a 5-year old *P.taiwaniana* plantation produced up to 226.03 m^3/ha of timber (Rin, 1979). The timber is light yet strong, dries easily, has a beautiful grain, does not warp or crack making it useful in the manufacture of furniture, toys, plywood and crates. Its outstanding resonance qualities make it suitable for musical instruments (Chinese Academy of Forestry, 1986; Burger, 1989). There is a potential export market and prices of US$ 300 per m^3 delivered in Japan are fetched (Donald, 1990). There is a need to expand the demand

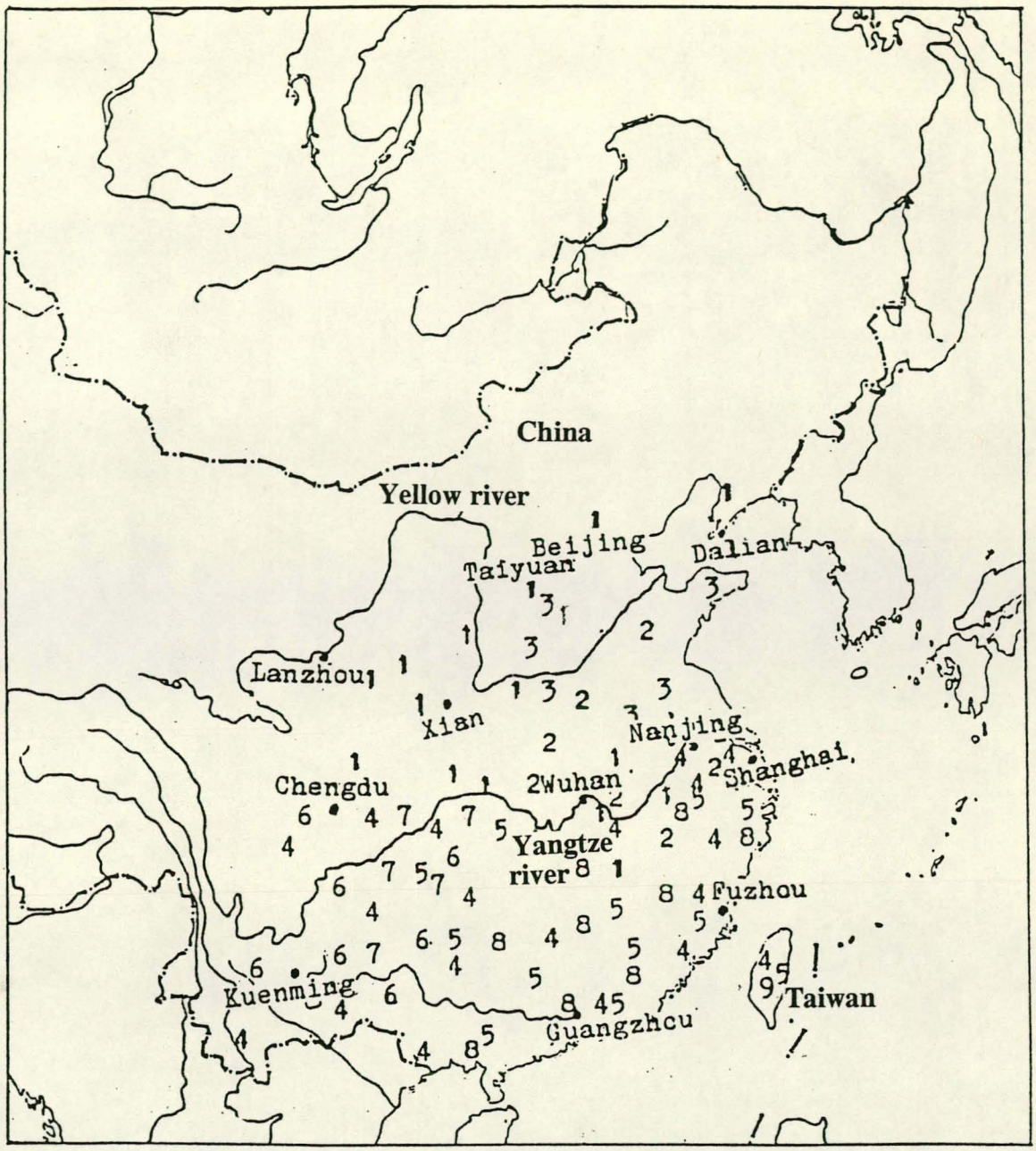


Fig. 1. 1. Distribution of various *Paulownia* species. (1) *P.tomentosa*, (2) *P.elongata*, (3) *P.catalpifolia*, (4) *P.fortunei*, (5) *P.kawakamii*, (6) *P.fargesii*, (7) *P.albiphloea*, (8) *P.australis*, (9) P.*taiwaniana* (after Chinese Academy of Forestry, 1986)

of the domestic market and to develop other overseas markets (Rin, 1975; Donald, 1990). Hardie *et al.* (1989) believe that the Japanese market will continue to expand sufficiently to absorb a modest US export of *Paulownia* timber for the foreseeable future.

Witches' broom disease is the most important factor limiting increased production. Most areas of China, Japan and Taiwan are seriously affected by this disease. It reduces tree growth by 20 - 80 % (Chinese Academy of Forestry, 1986; Iizuka & Funayama, 1984). The worst example of the effect of the disease has occurred in Taiwan. Almost 80 % of the Taiwanese plantations were infected within two years of the identification of the disease in 1974, virtually destroying the wood production industry of *Paulownia* in Taiwan (Chang *et al.*, 1978; Ying, 1978).

1.3 Disease

The major diseases of *Paulownia* are witches' broom, anthraconose, *Sphaceloma paulowniae* and damping-off disease. Witches' broom is an infectious disease and affects trees of all ages, unlike the other three diseases which affect only seedlings and young trees. It is wide spread throughout China (Chinese Academy of Forestry, 1986), Japan, Korea (Shiozawa *et al.*, 1979) and Taiwan (Chang *et al.*, 1978) and causes serious damage to *Paulownia* plantations. Growth is suppressed in infected trees followed by decline and premature death . Lumber of infected trees is of poor quality (Doi & Asuyama, 1981).

Witches' broom of *Paulownia* was first observed around 1880 in the southern district of Japan and the pathogen was first shown to be a mycoplasma-like organism (MLO) in 1967 (Doi & Asuyama, 1981). The vectors transmitting MLO were confirmed as the insects *Empoasca flavescens*, *Nesidiocoris tenuis*, *Gampscoris pulchellus*, *Halyomorpha*

halys and *H.picusin* in China (Chinese Academy of Forestry & Honan County Forestry Bureau ,1978; Jin *et al.*, 1981; Yuan, 1984) and *Halyomorpha mista* in Japan (Shiozawa *et al.*, 1979).

MLO closely resemble mycoplasmas in morphology and ultrastructure. Attempts to culture MLO in artificial media have failed and thus prevented its characterization by biochemical and serological methods (Freundt, 1981).

No cultivated species exhibiting immunity or resistance to the disease have been found (Doi & Asuyama, 1981). *P.tomentosa*, *P.fargesii* and *P.kawakamii* are equally susceptible. Doi and Asuyama (1981) suggested the following procedures to control the disease: (1) Disease-free seedlings should be selected for planting. (2) Rogue out diseased individual trees when symptoms appear. (3) Infected saplings can be cured by a trunk injection of tetracycline antibiotics. Eradication of the insect vectors by the use of insecticides to prevent the spread in field plantings is thought to be impractical, but may be helpful as a control method in nurseries. Yuan (1984) found *P.elongata* and *P.catalpifolia* to be the most susceptible species and *P.kawakamii* the least susceptible in natural stands of eight species of *Paulownia*. A *P.fortunei* tree exhibiting witches' broom resistance has been found in Taiwan (Su & Tsai, 1983), and a resistant gene in a hybrid *Paulownia* in China (Yang *et al.*, 1989). Wang *et al.* (1982) compared the hormone contents between healthy and infected trees of *P.elongata* and *P.catalpifolia*. Diseased trees had higher cytokinins and lower IAA in levels than healthy trees. He suggested that the morphogenesis of witches' broom is controlled by hormonal interactions.

1.4 Conventional breeding

Paulownia is heterozygous. Natural hybrids exist in areas where more than one species occur. Systematic interspecific crosses have been made and some superior hybrids have been selected. The inflorescence is a cyme of two to five flowers. *Paulownia* starts to flower from the second to sixth year after planting, but precocious flowering on seedling material does occur (Chinese Academy of Forestry & Honan County Forestry Bureau, 1978; Donald, personal communication 1992). GA_3 causes trees to flower at a younger age (Iizuka, 1985). Breeding is therefore easier than for most other tree species. Another advantage is the ease of propagating from root cuttings. Cloning however increases the risk of spreading the MLO disease.

1.5 Improvements by plant cell and tissue culture

Despite the relative ease to improve the population by conventional breeding methods, plant cell and tissue culture techniques can complement a breeding programme. The advantages of these techniques have been pointed out by Durzan (1985) as shown in Table 1. 1.

Plant cell and tissue culture of woody plants is difficult and the establishment of cell suspension, induction of somatic embryos, cell fusion and identification of desired genes and indeed micropropagation of mature trees particularly so (Hackett, 1987; Bonga, 1987; Thorpe, *et al.*, 1990). Once the micropropagation system of a specific line has been established, the higher cost of plantlet production becomes a problem (Boulay, 1987; Levin & Vasil, 1989). The development of mass production from somatic embryogenesis and robotic systems may reduce production cost (Boulay, 1987; Durzan, 1988; Levin & Vasil, 1989; Fijita, 1989), but this still appear some years in the future. Cell and tissue culture of *Paulownia* can complement conventional breeding in the following ways:

Table 1. 1. Comparison of properties of two breeding systems for the propagation of trees 1). conventional methods and 2). plant cell tissue culture techniques (after Durzan 1985)

Factor	Tree	Cell
Growth cycle	Decades to centuries	25-26 hours
Size	Up to 75 m high	50-100 μm
Space	10 acres of seed orchard	10 liter suspension culture
Numbers to mutation	10^7 trees	10^7 cells
Time to produce embryos	8-13 years (seeds)	1-2 years (somatic embryos)
Seed production predictability	Variable seed years	Controlled production on demand
Propagation	Cuttings, grafting, rooting	Organogenesis or embryogenesis
Ploidy	Haploids difficult to produce	Haploids easily cultured but more research needed
Flexibility in breeding	Barriers to cross-ability	Gene transformation can be attempted
Types of breeding system: mutation, inbreeding, hybridization, backcross, selection	Hybrids possible for certain species, require 8-10 year generation times, selection most applible	All breeding systems can be exploited cell and protoplasts, field test required for certification of quality and "true to type"
Cost to manittain system	$1,500/year/ha	$1,500 per liter (estimate)
Goals	Improved seed in quantity	Mass propagated elite hybrids

1. Multiplication or preservation of MLO-free superior clones by micropropagation from meristem tip or callus culture to replace root cuttings in MLO infection areas. Maia *et al.* (1973) and Maia and Beck (1976) have demonstrated that MLOs can be eliminated through meristemtip culture. Petru and Ulrychova (1975), Ulrychova and Petru (1975), Fedotina and Krylova (1976), Jacoli (1978) and Möllers and Sarkar (1989) obtained MLO-free plantlets from callus cultures.

2. Selection for plant variation such as disease resistance, heavy metal, salt, and cold tolerance etc. by cell cultures (Semal, 1986; Bajaj, 1990).

3. Regeneration of cells and protoplast for further cell fusion and gene transformation to obtain MLO-resistant plants from other genotypes. For instance, it is possible to obtain the MLO-resistant plants by cell fusion with some crops such as MLO-resistant *Oryza* or transformation of an insect resistant gene which has been identified in *Oryza* (Russell, 1978).

A breeding programme combining conventional methods and cell and tissue culture of *Paulownia* is outlined in Fig. 1. 2.

2. ORGANOGENESIS

In vitro organogenesis has been achieved in over 1000 plant species through empirical selection of the explant, medium composition and control of the physical environment (Brown & Thorpe, 1986). Organogenesis begins with the production of a unipolar bud primordium from a group of cells which develops into a leafy shoot, and which can root to form a plantlet. The other pathway is from embryogenesis which differs from

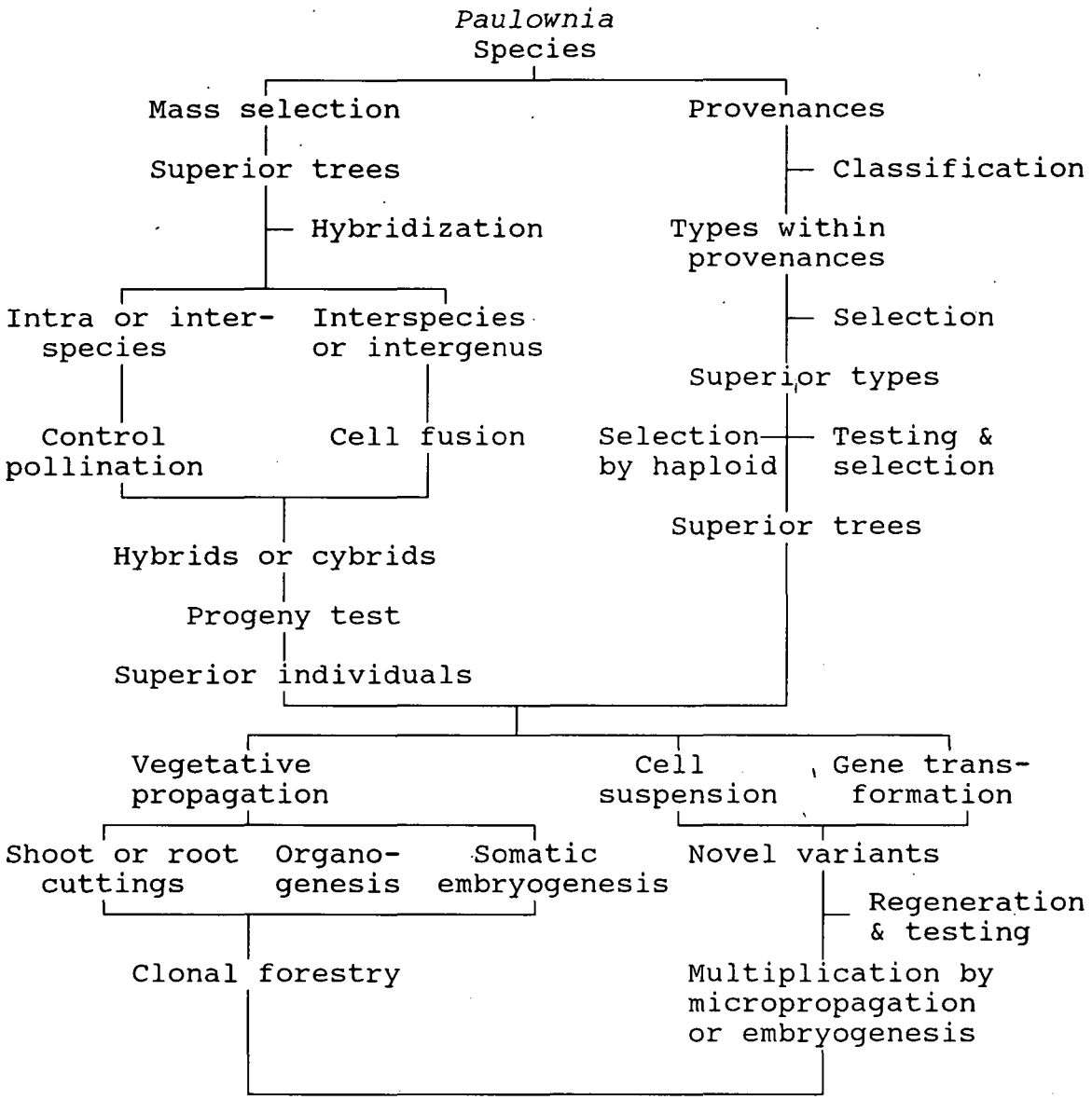


Fig. 1. 2. Breeding programme for combining conventional methods and plant tissue and cell culture of *Paulownia* (conventional methods are after Chinese Academy of Forestry, 1986)

organogenesis in that a single cell forms a bipolar embryo-like structure which can form a new individual plantlet. Only about 100 species have been successfully regenerated through embryogenesis (Terzi & Loschiavo, 1990).

Four species and one hybrid of *Paulownia* have been regenerated *in vitro* (Table 1. 2). The medium used was MS medium (Murashige & Skoog, 1962) with varying macrosalt concentrations. Hormones included various combinations of IAA and kinetin or BA, NAA or IBA and BA, or BA alone. NAA and BA combinations were more effective in inducing adventitious bud formation than other hormone combinations. Somatic variation of plantlets regenerated from *P.tomentosa* was significant (Jagannathan & Marcotrigiana, 1986). BA alone did not prevent callus formation in *P.elongata*, *P.taiwaniana* and *P.tomentosa* which was the reason for the variation (Ogura, 1990).

Burger *et al.*(1985) found that survival of nodal cultures of *P.tomentosa* was related to season. Nodes collected in June (summer) gave a 92 % survival rate compared to 0 to 27 % obtained for those from early or late April (spring). Yang *et al.* (1990) obtained 99 % survival rate in the culture of sprouting lateral buds of twigs after removing twig tips. Higher concentrations of BA up to 15 mg l^{-1} were necessary for shoot formation of adult trees of *P.taiwaniana* (Yang *et al.*, 1990). But for nodal explants of *P.tomentosa* BA 5 mg l^{-1} and NAA 0.5 mg l^{-1} was needed to induce shoots from axillary buds (Burger *et al.*, 1985).

Rooting of shoots obtained from juvenile tissues for *P.elongata*, *P.taiwaniana* and *P.tomentosa* were induced readily on MS medium without hormones. Cuttings rooted equally well in a mixture of peat, vermiculite and compost in a greenhouse (Marcotrigiano & Stimart, 1983; Ho *et al.*, 1988; Chang & Donald, 1992). Mature IBA or in a greenhouse after dipping basal parts of cuttings in 1000 mg l^{-1} IBA for 15

Table 1. 2. Regeneration of organ and tissue culture for *Paulownia* species

Species	Explants	Media	Hormones mg l^{-1}	Results	References
elongata	Shoot tip, node, internode & leaf of plantlet	MS	BA 0.1-5 NAA 0.01, 1 & BA 0.1-5	10 ABs[1]/shoot tip on BA 1 only 3-50 ABs/explant on NAA & BA	Chang & Donald, 1992
elongata x *tomentosa*	Immature embryos	MS ½MS ½MS	NAA 0.3 & BA 4.5 NAA 0.1 & BA 2-3 NAA 0.8	Primary culture Shoot proliferation Root formation	Huang, 1985
fortunei	?	MS ½MS	IAA 1 & BA 4 0	Primary and shoot proliferation. Root formation.	Yang, 1982
taiwaniana	Node & internode of seedlings	½MS	IAA 2,4 & Kin[2] 1 2	callus in dark, 2-4 ABs/nodes in light	Fan & Hu, 1976 Fu, 1978
	Shoot tip, node, internode, leaf of plantlets	½MS MS	NAA 0.2-3 & BA or Kin 0.1-10	>30 ABs/internode, 9 ABs/bud, 2 ABs/node, root *in vivo*	Ho *et al.*, 1988
	Lateral buds from 8 year-old tree	1, ½, 1/3 MS	BA or Kin 1-15 NAA, IBA 0-4	50 ABs/bud on BA 15, Root on IBA 2	Yang *et al.*, 1990
tomentosa	cotyledon & hypocotyl shoot tips	MS MS	IAA 0-10, & Kin 0-3 IBA 0-10 & BA 0-10, GA 0.1	Root in cotyledon 3.6 ABs/hypocotyl 10.5 shoots/AB	Marcotrigiano & Stimart, 1983
	Hypocotyl	MS	IAA 3 & Kin 3	1/21 plantlets had mixploid & 5 phenotypic variants	Jagannathan & Marcotrigiana, 1986
	Nodes of 15 year-old trees	½MS	BA 1-10 & NAA 0.1, 0.5,1 IBA 0.5,1	92% nodes induced axillary buds, 100% root formation	Burger *et al.*, 1985

[1]ABs: adventitious buds, [2]Kin: kinetin

shoots of *P.tomentosa* also rooted well on ½MS medium containing 0.5 or 1 mg l^{-1} seconds. However, mature shoots of *P.taiwaniana* were difficult to root on solid medium, rooting occurred on filter paper soaked in liquid MS medium containing 4 mg l^{-1} IBA (Yang *et al.*, 1990).

3. EMBRYOGENESIS

Somatic embryogenesis will be the most important method of micropropagation in the next few years, especially somatic embryogenesis in cell suspensions. Millions of embryos can be produced and encapsulated to form artificial seeds (Bajaj, 1986).

The only report on somatic embryogenesis of *Paulownia* is by Radojevic (1979). He obtained somatic embryos from fertilized ovules and germinating seed embryos of *P.tomentosa*. The culture medium was MS medium containing 0.7 % agar, 200 mg l^{-1} casein hydrolysate, 100 mg l^{-1} myoinositol, 2 mg l^{-1} thiamine, 5 mg l^{-1} nicotinic acid, 2 mg l^{-1} adenine, and 10 mg l^{-1} pantothenic acid. Ovules attached to the placenta were cultured on medium containing 1 mg l^{-1} 2,4-D. After they grew to 2-3 times in size, single ovule were transferred to new medium and then subcultured on the same medium every month. After three month numerous embryos formed from ovule derived callus. Callus maintained its embryogenic capacity for 4 years by regular subculturing. A histological examination showed embryogenic nodules of various sizes, distributed throughout the large cells of parenchymatous callus tissue. Proembryos observed on the superficial layers of the nodules were thought to originate from conspicuous, single cells, near the surface of callus. Somatic embryos from ovule callus failed to develop into plantlets.

In contrast, somatic embryos obtained from germinating embryos cultured on a medium containing 0.1-1 mg l^{-1} IAA and 0.1-1 mg l^{-1} kinetin did develop into plantlets. However, this embryogenic callus gradually became necrotic after 3 months in culture (Radojevic, 1979).

4. CALLUS CULTURE

In plant cell cultures, callus is largely an unorganized, proliferating mass of parenchyma cells. With age such callus tissue may show meristematic islands or strands of individual or groups of tracheids and pigmented cells (Constabel, 1984). After prolonged culture callus tissues may undergo endomitosis, chromosome loss, translocations, poliploidy, aneuploidy, gene amplification, cryptic chromosome rearrangements, somatic gene rearrangement, mutations and other genetic changes. It is a rich source of genetic diversity to increase the genetic variability for breeding selection (Bajaj, 1986). Plantlets from hypocotyl callus of *P.tomentosa* have proved highly variable (Jagannathan & Marcotrigiano, 1986). Although mixploidy and aberrant seedlings resulted in reduced growth rate, it has been suggested that poliploid trees may be important for tree improvement. For instance, polyploid *Populus* trees grow faster than the diploids (Bajaj, 1986). Another advantage of callus cultures is the enormous number of plants that can be produced from callus-derived cell suspensions.

Fu (1978) reported that firm, white callus derived from seedling stems of *P.taiwaniana* cultured on ½MS medium containing 5 mg l^{-1} IAA, 2.5 mg l^{-1} kinetin, and 15 % coconut milk had the capacity to form adventitious shoots. After several subcultures the ability to regenerate decreased. The other report on this subject is that of Radojevic (1979) which has already been discussed under embryogenesis. Nothing has been

reported on the induction of friable callus for cell suspension, and suspension cultures from embryogenic callus.

5. ANTHER CULTURE

Anther culture to produce haploids may be utilized to facilitate the detection of mutations and the recovery of unique recombinants, since there in no masking of recessive alleles. Furthermore, doubling of the chromosome number of haploids offers a method for the rapid production of homozygous plants, which in turn can be used for producing inbred lines for hybrid production (Bajaj, 1983). Androgenesis has been reported in some 171 species, of which many are important crop plants (Hu & Zeng, 1984) and in 24 woody species callus, somatic embryos or plantlets were obtained from anther culture (Bajaj, 1983).

Yang (1988) studied the relationship of the development of flower buds and pollen of *P.taiwaniana*. Anthers of different developmental stages were cultured in MS and Nitsch and Nitsch medium (Nitsch & Nitsch, 1969) containing 3-9 % sucrose and combinations of NAA and BA or kinetin. He found that anthers in 60 % of the cultures in the early and late uninucleate stages produced callus when cultured on MS medium containing 3 % sucrose and 3 mg l^{-1} NAA plus 5 mg l^{-1} BA. However, sections of the callus revealed that the callus originated from anther tissues and not pollen..

6. PROTOPLAST ISOLATION, CULTURE AND FUSION

Genes can be transferred with conventional breeding methods, protoplast fusion, agrobacterium-mediated methods, and introduction of foreign genes into protoplasts (Ahuja, 1988). Hybridization usually requires backcrossing to transfer one or a few genes from one species to another. This procedure involves several generations to introduce a desirable gene into the second species and is limited to those plant species that are sexually compatible. Protoplasts have been shown to provide an excellent method for genetic manipulation. Protoplast cultures result in plantlets with a wide range of genetic variation from which to select (Pelletter *et al.*, 1988). Protoplast fusion overcomes the barrier of sexual incompatiblity allowing the creation of somatic hybrids and cybrids. The newer techniques of electrofusion which increases the frequency of fusion (Zimmermann & Scheurich, 1981), and flow cytometry which allows the rapid selection of fusion cells are especially effective (Galbraith, 1984; Glimelius, 1987; Göhde *et al.*, 1990). Protoplasts can also be used for direct gene transfer without the concomitant transfer of undesirable traits (Wilk-Douglas *et al.*, 1986) which would provide a revolutionary method for tree breeding (McCown *et al.*, 1991).

Protoplast culture of forest trees is still difficult (Ahuja, 1982; 1984; 1986; 1988; David, 1987; McCown & Russell, 1987). To date, protoplast regeneration was successful from embryogenic cell suspensions of conifer species (Attree *et al.*, 1987; Gupta & Durzan, 1987) and few species of hard-woods (McCown & Russell, 1987; Patat-Ochatt, *et al.*, 1988; Ito *et al.*, 1990; Ochatt, 1990; Park & Son, 1992), while somatic hybrid plants of some woody species have been obtained (Ohgawara *et al.*, 1985; Ito *et al.*, 1986; Grosser, *et al.*, 1988a and b). Foreign gene transfer into trees and subsequent regeneration of transformed plants has been reported in *Populus*, *Juglans* and *Malus* (McCown *et al.*, 1991)

Protoplast isolation of *P.fortunei* and *P.taiwaniana* from mesophylls of 1 year-old seedlings and protoplast fusion between *P.taiwaniana* and *Populus euramericana* cv. I-45/51 have been reported (Saito, 1980a and b; Yang *et al.*, 1989), but regeneration was not achieved. High yields of protoplasts (2 x 10^7 protoplasts per gram leaves) were obtained when leaves were collected in summer, the epidermis was removed and incubated at 30°C in Saito or MS medium, without NH_4NO_3 solution, but containing digestive enzymes (Yang *et al.*, 1989).

7. STUDY OBJECTIVES

The main objective of this study was to establish the regenerative ability on tissue, cell and protoplast level for the genus *Paulownia*. For this purpose three species viz *P.fortunei*, *P.kawakamii* and *P.tomentosa* which represent three sections of the genus were selected. *P.taiwaniana* was also included because much of the *in vitro* studies on *Paulownia* have been done on this species and it was therefore useful for comparative purposes. The specific objectives included:

(i) To establish the organogenetic and embryogenic potential in explants from various plant parts and as affected by media composition and cultural conditions.

(ii) To establish callus cultures from different explant types and to assess the regenerative ability of the callus by manipulation of the culture media.

(iii) To establish cell suspensions and determine the conditions for long term maintenance of the cell cultures and to determine the requirements for the regeneration of plants from suspension cells.

(iv) To develop procedures for the isolation and culture of protoplasts and the regeneration of plants from protoplasts.

8. REFERENCES

Ahuja, M.R., 1982. Isolation, culture and fusion of protoplasts: problems and prospects. Silvae Genet. 31:66-77

Ahuja, M.R., 1984. Protoplast research in woody plants. Silvae Genet. 33:32-37

Ahuja, M.R., 1986. Perspectives in plant biotechnology. Curr. Sci. 55:217-224

Ahuja, M.R., 1988. Gene transfer in forest trees. In: Hanover, W., Keathley, D,E. (Eds.) Genetic manipulation of woody plants. (pp. 25-41) Plenum Press, New York

Attree, S.M., Bekkaoui, F., Dunstan, D.I. and Fowke, L.C., 1987. Regeneration of somatic embryos from protoplasts isolated from an embryogenic suspension culture of white spruce (*Picea glauca*). Plant Cell Rep. 6:480-483

Bajaj, Y.P.S., 1983. *In vitro* production of haploids. In: Evans D.A., Sharp W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture. Vol.1 (pp. 228-287) Macmillan Publishers Co., New York

Bajaj, Y.P.S., 1986. Biotechnology of tree improvement for rapid propagation and biomass energy production. In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry 1: Trees I. (pp. 1-23) Springer-Verlag, Berlin

Bajaj, Y.P.S., 1990. Somaclonal variation - origin, induction, cryopreservation, and implications in plant breeding. In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry 2: Somaclonal variation in crop improvement. (pp 3-48) Springer-Verlag, Berlin

Bonga, J.M., 1987. Clonal propagation of mature trees: problems and possible solutions. In: Bonga, J.M. and Durzan D.J. (Eds.) Cell and tissue culture in forestry Vol.1: General principles and biotechnology. (pp. 249-271) Martinus Nijhoff Publishers, The Netherlands

Boulay, M., 1987. Conifer micropropagation: applied research and commercial aspects. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry Vol.3: Plant regeneration and genetic variability. (pp. 185-206) Martinus Nijhoff Publishers, The Netherlands

Brown, D.C., and Thorpe, T.A., 1986. Plant regeneration by organogenesis. In: Vasil, I.K. (Ed.) Cell culture and somatic cell genetics of plants. Vol.3 (pp. 49-65) Academic Press, New York

Burger, D.W., 1989. Empress tree (*Paulownia tomentosa* Steud.). In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry 5. trees II. (pp. 359-369) Springer-Verlag, Berlin

Burger, D.W., Liu, L. and Wu, L., 1985. Rapid micropropagation of *Paulownia tomentosa*. HortSci. 20(4):760-761

Chang, S.H. and Donald, G.M.D., 1992. *In vitro* organogenesis and plantlet formation of *Paulownia elongata*. South African For. J. (in press)

Chang, Y,C., Su, H.J., and Wu, R.Y., 1978. Preliminary study on paulownia witches' broom in Taiwan. Proc. ROC US Cooperative Semin. Mycoplasma Dis. Plants, March 27-31 (pp. 127-137) Nat. Sci. Council, Taipei

Chinese Academy of Forestry, 1986. *Paulownia* in China: cultivation and utilization. Asian Network of Biol. Sci. & Nat. Develop. Res. Center, Canada

Chinese Academy of Forestry and Honan County Forestry Bureau, 1978. Research on *Paulownia*. (pp. 266) Chinese For. Publish.

Constabel, F., 1984. Callus culture: induction and maintenance. In: Vasil, I.K. (Ed.) Cell culture and somatic cell genetics of plants. Vol.1: Laboratory procedures and their applications (pp. 27-35) Academic Press, New York

David, A., 1987. Conifer protoplasts. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry Vol.2: Cell growth, nutrition, cytodifferentiation and cryopreservation. (pp. 2-15) Martinus Nijhoff Publishers, The Netherlands

Doi, Y. and Asuyama, H., 1981. *Paulownia* witches' broom disease. In: Maramorosch, K. and Raychaudhuri, S.P. (Eds.) Mycoplasma diseases of trees and shrubs (pp. 135-145) Academic Press, New York

Donald, D.G.M., 1990. *Paulownia* - the tree of the future. South African For. J. 154:94-98

Durzan, D.J., 1985. Cell and tissue culture in forest industry. In: Bonga, J.M. and Durzan, D.J. (Eds.) Tissue culture in forestry. (pp. 36-71) 2nd edition, Martinus Nijhoff Publishers, The Netherlands

Durzan, D.J., 1988. Applications of cell and tissue culture in tree improvement. In: Ciba Found. Symp. (Ed.) Applications of plant cell and tissue culture. (pp. 36-58) John Wiley & Sons Ltd., UK

Fan, M.L. and Hu, T.W., 1976. Plantlets from *Paulownia* tissue cultures. Bull. of Taiwan For. Res. Inst. No. 286:1-6

Fedotina, V.L. and Krylova, N.V., 1976. Ridding tobaccos of the mycoplasmic infection big bud by the method of tissue culturing. Dokl. Bot. Sci. 228:49-51

Fijita, N., 1989. Application of robotics to masspropagation system. In Vitro Cell. Dev. Biol. 25(3):22A

Freundt, E.A., 1981. Isolation, characterization, and identification of Spiroplasmas and MLOs. In: Maramorosch, K. and Raychaudhuri, S.P. (Eds.) Mycoplasma diseases of trees and shrubs. (pp. 135-145) Academic Press, New York

Fu, M.L., 1978. Plantlets from *Paulownia* tissue culture. Gardens Bull. (Singapore) 31:61-66

Galbraith, D.W., 1984. Flow cytometric analysis of the cell cycle. In: Vasil, I.K. (Ed.) Cell culture and somatic cell genetics. (pp. 765-777) Academic Press, New York

Glimelius, K., 1987. Enrichment of somatic hybrids with flow cytometry. Int. Assoc. for Plant Tissue Cult. 52:2-10

Göhde, M., Boon, E.J. and Tan, M.M.C., 1990. A new fluorescence activated sorting technology for plant protoplast. Abstracts VIIth Int. Cong. on Plant Tissue & Cell Cult. (p. 14) Amsterdam

Gupta, P.K. and Durzan, D.J., 1987. Somatic embryos from protoplasts of loblolly pine proembryonal cells. Bio/Tech. 5:710-712

Grosser, J.W., Gmitter, F.G. and Chandler, J.L., 1988a. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia distcha*. Theor. Appl. Genet. 75:397-401

Grosser, J.W., Gmitter, F.G. and Chandler, J.L., 1988b. Intergeneric somatic hybrid plants of *Citrus sinensis* cv. Hamlin and *Poncirus trifoliata*. Plant Cell Rep. 7:5-8

Hackett, W.P., 1987. Juvenility and maturity. In: Bonga, J.M. and Durzan D.J. Eds. Cell and tissue culture in forestry Vol.1: General principles and biotechnology. (pp. 216-231) Maritinus Nijhoff Publishers, The Netherlands

Hardie, I., Kundt, J.F. and Miyasaka, E., 1989. Economic feasibility of US *Paulownia* plantations. J. of For. 87:19-24

Ho, C.K., Chang, S.H. and Yang, J.C., 1988. Tissue culture of *Paulownia taiwaniana* from juvenile tissues. Sym. of Tree Improv. & Tissue Culture in Taiwan. (pp. 97-113) Exp. For. of Nat. Taiwan Univ.

Howlett, D., 1975. Forestry in the future of Brazil. Am. For. 81:(11):14-17, 44-45

Hu, H. and Zeng, J.Z., 1984. Development of new varieties via anther culture. In: Ammirato, P.V., Evans, D.A., Sharp, W.R. and Yamada, Y., (Eds.) Handbook of plant cell culture Vol.3: Crop species (pp. 65-90) Macmillan Publishers Co., New York

Hu, S.Y., 1959. A monograph of the genus *Paulownia*. Quart. J. Taiwan Museum XII No 1 & 2:1-54

Huang, C., 1985. *In vitro* culture and plantlet regeneration from immature embryo of *Paulownia elongata* x *tomentosa*. Plant Physiol Commun. 1:39 (in Chinese). In: Chen Z, Evans, D.A., Sharp, W.R., Ammirato, P.V. and Sondahl, M.R. (Eds.) 1990. Handbook of plant cell culture Vol.6: Perennial crops. (pp. 39) McGraw-Hill Publishing Company, USA

Iizuka, M., 1985. Induction of flower bud formation (by gibberelin GA_3) in young tree of *Paulownia*. Bull. For. & For. Prod. Res. Inst. 331:155-165 (Japan)

Iizuka, M. and Funayama, E., 1984. Influence of witches' broom on tree growth of *Paulownia*. Bull. For. & For. Prod. Res. Inst. 331:147-154 (Japan)

Ito, K., Doi, K., Tatemichi, Y. and Shibata, M., 1990. Plant regeneration from protoplasts of *Eucalyptus*. In: Abstracts VIIth Int. Congress on Plant Tissue and Cell Culture (pp. 19) Amsterdam

Ito, K., Tatemichi, Y. and Shibata, M., 1986. Regeneration of poplar-like plants from fusion between *Populus* (hybrid) and *Hibiscus*. Proc. VI Int. Congr. Plant Tissue and Cell Culture, Univ. Minnesota, MN, Abstr, pp. 389

Jacoli, G.G., 1978. Sequential degeneration of mycoplasma like bodies in plant tissue cultures infected with aster yellows. Can. J. Bot. 56:133-140

Jagannathan, L., Marcotrigiano, M., 1986. Phenotypic and ploidy status of *Paulownia tomentosa* trees regenerated from cultured hypocotyls. Plant Cell, Tissue & Organ Cult. 7:227-236

Jin, K.S., Liang, C.J. and Deng, D.L., 1981. A study of the insect vectors of witches' broom in *Paulownia* trees. Linye Keji Tongxun 12:23-24

Levin, R. and Vasil, I.K., 1989. Progress in reducing the cost of micropropagation. Newsletter, Int. Assoc. For Plant Tissue Cult. 59:2-12

Lin, T.P. and Wang, Y.S., 1991. *Paulownia taiwaniana*, a hybrid between *P.fortunei* and *P.kawakamii* (Scrophulariaceae). Plant Systematics and Evolution in press.

Maia, E. and Beck, D., 1976. Cultures d'organes *in vitro* et regeneration de plantes infectees par des mycoplasmes. Ann. Phytopathol. 8:303-306. In: Möllers, C. and Sarkar, S., 1989. Regeneration of healthy plants from *Catharanturs roseus* infected with mycoplasma-like organisms through callus culture. Plant Sci. 60:83-89

Maia, E., Bettachini, B., Beck, D., Bernard, P. and Maia, N., 1973. Contribution a l'amelioration de l'etat sanitaire du Labandin, clone 'Abrial'. Ann. Phytopathol. 5:115-

124. In: Möllers, C. and Sarkar, S., 1989. Regeneration of healthy plants from *Catharanturs roseus* infected with mycoplasma-like organisms through callus culture. Plant Sci. 60:83-89

Marcotrigiano, M. and Stimart, D.P., 1983. *In vitro* organogenesis and shoot proliferation of *Paulownia tomentosa* Steud (Empress tree). Plant Sci. Lett. 31:303-310

McCown, B.H. and Russell, J.A., 1987. Protoplast culture of hard woods. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry. Vol.2 (pp. 16-30) Martinus Nijhoff Pub., Netherlands

McCown, B.H., McCabe, D.E., Russell, D.R., Robison, D.J., Barton, D.A. and Raffa, K.F., 1991. Stable transformation of Populus and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep. 9:590-594

Möllers, C. and Sarkar, S., 1989. Regeneration of healthy plants from *Catharanturs roseus* infected with mycoplasma-like organisms through callus culture. Plant Sci. 60:83-89

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479

Nitsch, J.P. and Nitsch, C., 1969. Haploid plants from pollen grains. Science 163:85-87

Ochatt, S.J., 1990. Plant regeneration from root callus protoplasts of sour cherry (*Prunus cerasus* L.). Plant Cell Rep. 9:268-271

Ogura, H., 1990. Chromosome variation in plant tissue culture. In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry II: Somaclonal variation in crop improvement I (pp. 49-84) Springer-Verlag, Berlin

Ohgawara, D., Kobayashi, S., Oghawara, E., Uchimiya, H. and Ishii, S., 1985. Somatic hybrid plants obtained by protoplast fusion between *citrus sinensis* and *Poncirus trifoliata*. Theor. Appl. Genet. 71:1-4

Park, Y.G. and Son, S.H., 1992. *In vitro* shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (*Populus nigra* x *P.maximowiczii*). Plant Cell Rep. 11:2-6

Patat-Ochatt, E.M., Ochatt, S.J. and Power, J.B., 1988. Plant regeneration from protoplasts of apple rootstocks and scion varieties (*Malus* x *domestica* Borkh.). J. Plant Physiol. 133:460-463

Pelletter, G., Primard, C., Ferault, M., Vedel, F., Chetrit, P., Renarc, M. and Delourme, R., 1988. Use of protoplasts in plant breeding: cytoplasmic aspects. Plant Cell, Tissue & Organ Cult. 12:173-180

Petru, E. and Ulrychova, M., 1975. Persistence and spread of mycoplasma in axenic callus tissue of tobacco in the presence of kinetin and IAA in the nutrient medium. Biol. Plant. 17:352-356

Radojevic, L., 1979 Somatic embryos and plantlets from callus cultures of *Paulownia tomentosa* Steud. Z Pflanzenphysiol. 91:57-62

Rin, U.C., 1975. The trend of wood production for *Paulownia* in world. Taiwan For. 1(9):9-15

Rin, U.C., 1979. Silvicultural studies on Taiwan Paulownia (*Paulownia taiwaniana* Hu et Chang) in Taiwan. (pp. 228) Research Bull. No.178:1-228 Dep. of For. Nat. Chung Hsing Univ (in Chinese).

Russell, G.E., 1978. Plant breeding for pest and disease resistance. (pp. 199-208) Butterworths & Co Ltd., England

Saito, A., 1980a. Isolation of protoplasts from mesophyll cells of *Paulownia* and *Populus*. Bull. For. & For. Prod. Res. Inst. 309:1-6

Saito, A., 1980b. Fusion of protoplasts isolated from somatic cells of tree species. Bull. For. & For. Prod. Res. Inst. 309:7-12

Semal, J., 1986. Somaclonal variations and crop improvement. (pp. 277) Martinus Nijhoff Pub.

Shiozawa, H., Yamashita, S., Doi, Y., Yora, K. and Asuyama, H., 1979. Trial of transmission of paulownia witches' broom by two species of bug, brown marmorated stink bug and brown-winged green bug, observed on paulownia. Ann. Phyopathol. Soc. Jap. 45:556

Su, H.J. and Tsai, L.S., 1983. The resistance of *Paulownia* against witches' broom disease. Quar. J. Chin. For. 16(2):187-202

Terzi, M. and Loschiavo, F., 1990. Somatic embryogenesis. In: Bhojwani, S.S., (Ed.) Plant tissue culture: Applications and limitations. Developments in crop Science 19 (pp. 54-66) Elsevier Sci. Publishers, Netherlands

Thorpe T.A., Harry, I.S. and Kumar P.P., 1990. Application of micropropagation to forestry. In: Debergh, P.C. and Zimmerman R.H. (Eds.) Micropropagation: Technology and application (pp. 311-336) Kluwer Academic Pub. Netherlands

Ulrychova, M. and Petru, E., 1975. Elimination of mycoplasma in tobacco callus tissues (*Nicotiana glauca* Grah) cultured in the presence of 2,4-D in the nutrient medium. Biol. Plant. 17:103-108

Wang, R., Wang, S.Z. and Sun, S.Q., 1982. Effect of hormones on the morphogenesis of witches' broom of *Paulownia* spp. Sci. Silvae Sinicae 17(3):281-286

Wilk-Douglas, M., Perani, L., Radke, S. and Bossert, M., 1986. The application of recombinant DNA technology toward crop improvement. Physiol. Plant. 68:560-565

Yang, J.C., 1988. Micropropagation, anther culture and protoplast isolation in *Paulownia* species. Nat. Sci. Council of Rep. of China, NSC-77-04090B-054-06 (in Chinese)

Yang, J.C., Chang, S.H. and Ho, C.K., 1990. Micropropagation of *Paulownia taiwaniana* from mature tissues. In: Dreyer, E., Aussenac, G., Bonnet-Masimbert, M., Dizengremel, P., Favre, J.M., Garrec, J.P., Le Tacon, F. and Martin, F. (Eds.) Forest Tree Physiology.(pp 165s-167s) Proc. of the Int. Sym. 25-30 Sep., 1988 Nancy, France

Yang, J.C., Ho, C.K., Chang, S.H. and Huang, F.H., 1989. The isolation of mesophyll protoplasts in *Paulownia taiwaniana*. In: Yang, J.C., Lin, T.P. and Wang, W.Y. (Eds.) Sino-American workshops on forest biotechnology and molecular biology. (pp. 51-60) Nat. Sci. Council, Rep. of China

Yang, N., 1982. Tissue culture and organogenesis of some woody plants in vitro. Plant Physiol. Commun. 4:23-27. (in Chinese). In: Chen, Z., Evans, D.A., Sharp, W.R., Ammirato, P.V. and Sondahl, M.R. (Eds.) 1990. Handbook of plant cell culture. Vol.6 (p. 39) McGraw-Hill Publishing Company

Ying, S.L., 1978. Witches' broom disease of *Paulownia* (P.*taiwaniana* Hu & Chang) in Taiwan. Taiwan For. Bureau (pp. 161-168)

Yuan, T.L., 1984. Some studies on witches' broom disease of *Paulownia* in China. Int. J. Tropic Plant Disease 2(2):181-190

Zimmermann, U. and Scheurich, P., 1981. High frequency fusion of plant protoplasts by electric fields. Planta 151:26-32

CHAPTER 2

SEED GERMINATION OF *PAULOWNIA* SPECIES

Key words: chemical solution, germination, sterilization

ABSTRACT

Germination of both mature and immature seeds for *P.fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* were stimulated by soaking seeds in a 3.5 % sodium hypochlorite solution. Pretreatment of the seeds with 70 % alcohol for 1 min before soaking in the sodium hypochlorite solution caused higher germination percentages than sodium hypochlorite treatment alone. MS medium inhibited germination of mature seeds, but germination of immature seeds was not affected to the same extent. Analyses of the effect of individual components of MS medium on the germination of *P.tomentosa* revealed that sucrose, the total salts and some macronutrients were the major inhibitory factors. *Paulownia* seeds should therefore be germinated in water solidified with 0.6 % agar before transfer to MS medium for *in vitro* growth.

1. INTRODUCTION

Seeds of *Paulownia* species require light exposure to germinate (Toole *et al.*, 1958; Borthwick *et al.*, 1964; Wang & Hong, 1979; Grubisic *et al.*, 1985 and 1988). This not withstanding dormancy percentages of *P.fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* varied significantly between different provenances and seed lot

collections but the dormancy could be broken by immersing the seeds in a 5 % sodium hypochlorite solution for 20 minutes (Donald, 1987). The use of 70 - 75 % alcohol is frequently used prior to sodium hypochlorite soaking to obtain contaminant-free cultures *in vitro* (Hu & Wang, 1983). These two sterile chemicals have been proved to stimulate germination of *Capsicum annuum* cv. Calwonder (Abdul-Baki, 1974), *Lycopersicon esculentum* cv. Potmac, *Hordeum vulgare* cv. Himalaya and *Lectuca sativa* cv. Grand Rapids (Emal & Conard, 1972), *Sorghastrum nutans* (Fieldhouse & Sasser, 1975), *Stipa viridula* (Frank & Larson, 1970), *Avena fatua*, *Polynomum convovulus* and *Saponaria vaccaria* (Hsiao, 1979a and b), and *Panicum capillare*, *Digitaria sanguinalis*, *D.ischaemum*, *Echinochloa crus-galli*, *Eragrostis cilianensis* and *Setaria faberi* (Taylorson & Hendricks, 1979). However, the sterilized seeds of *Paulownia* which were sown on MS medium (Murashige & Skoog, 1962) with agar germinated unsatisfactorily in the author's experiments. This study examines the possible factors (alcohol, hypochlorite, agar and MS medium) that could affect the seed germination of *Paulownia* species.

2. MATERIAL AND METHODS

2.1 Seed sources

Mature seeds of *Paulownia* were obtained from the following sources: seeds of *P.fortunei* collected from Taiwan University in 1991, *P.kawakamii* and *P.taiwaniana* were collected from Kukuan, Taiwan in 1985 and coldstored at $0^{o}C$ for 4 years at Taiwan Forest Research Institute (TFRI), and *P.tomentosa* introduced from Japan by TFRI in 1988. Seeds placed in polyethelene bags were stored at $4^{o}C$ in a refrigerator at the Department of Botany, University of Stellenbosch until used. Green entire

capsules containing immature seeds of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* were collected in 1992 from trees at the Department of Forestry , University of Stellenbosch.

2.2 Seed germination

2.2.1 Preparation of planting media, culture condition and experimental design

In this series of experiments, all aseptic work was conducted under a laminar flow bench, the pH of the germination media were adjusted to 5.7 and the germination media (except experiment 2.2.2) were autoclaved for 15 min at 121^{o}C. Treatments were replicated 4 times each with 100 seeds per treatment. Seeds were germinated in a culture room at 25^{o}C under 30 $\mu Em^{-2}s^{-1}$ fluorescent and incandescent light with a 16 hour photoperiod. A randomized complete block design was used. Germination percntages were recorded every 2 weeks during incubation. Analysis of variance was done following logit transformation of the data (Snedecor & Cochran, 1980), using General Linear Models (GLM) of SAS (SAS Institute Inc., 1982).

2.2.2 Effect of alcohol and sodium hypochlorite on germination of mature seeds

Mature seeds of *P.kawakamii* and *P.tomentosa* were subjected to the following treatments: (1) Control, seed untreated; (2) Seeds soaked in 70 % alcohol for 1 min and stirred occasionally, then rinsed 3 times with distilled water; (3) Seeds soaked in commercial bleach (3.5 % sodium hypochlorite) for 5, 15, 30, 60 and 120 min and stirred occasionally, then rinsed 3 times with distilled water; (4) Seeds soaked in 70 % alcohol for 1 min and then soaked into commercial bleach containing a drop of Tween 20 per 100 ml bleach for 15 min, followed by 3 rinses in distilled water.

Seeds were placed in 9-cm diameter Petri dishes containing 2 ml distilled water as described by Grubisic *et al.* (1985), and allowed to germinate.

2.2.3 Effect of water and MS medium with or without agar on the germination of mature seeds

Mature seeds of *P.fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* were sterilized as described in treatment (4) of experiment 2.2.2. Untreated seeds germinated in distilled water were included as a control. Seeds were placed in a 9-cm Petri dish containing 5 ml of either sterile distilled water or sterile MS medium both with or without 0.6 % Difco agar.

2.2.4 Effect of chemical composition of germinating medium on seed germination of *P.tomentosa*

Seeds of *P.tomentosa* were sterilized with alcohol and sodium hypochlorite as described in experiment 2.2.3. Seeds were placed in Petri dishes each containing 2 ml of the different fractions of MS medium listed in Table 2. 6. Sterile water was used as control.

2.2.5 Germination of immature seeds.

Immature seeds of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* were removed aseptically from capsules sterilized as described in experiment 2.2.3. The immature seeds were planted directly into Petri dishes containing 5 ml of distilled water, water with 0.6 % agar, liquid MS medium and MS medium with 0.6 % agar. For comparison immature

seeds were sterilized with alcohol and commercial bleach as previously described and planted in water, liquid MS medium and MS medium with 0.6 % agar.

3. RESULTS

3.1 Effect of alcohol and sodium hypochlorite on germination of mature seeds

Anova of germination percentages of *P.kawakamii* and *P.tomentosa* seeds treated with sterile chemicals is presented in Table 2. 1. Less than 20 % of seeds germinated for *P.kawakamii* when they were untreated or treated with alcohol (Table 2. 2). Germination varied between 22-28 % when soaked in sodium hypochlorite whereas 52 % seeds germinated when sterilized with alcohol and hypochlorite. Seeds of *P.tomentosa* germinated in greater numbers than *P.kawakamii*. Germination was between 72 and 75 % when sterilized with hypochlorite, and 85 % when treated with alcohol and hypochlorite. The treatment time with sodium hypochlorite had no effect on the germination for either species. However, soaking in hypochlorite in excess of 1 hour caused poor seedling growth (data not shown).

Table 2. 1. Anova of germination percentages of *P.kawakamii* and *P. tomentosa* seeds treated with alcohol, sodium hypochlorite and their combination.

Source of variances	Df	Mean squares	
		P.kawakamii	*P.tomentosa*
Replication	3	0.02	0.15
Treatments	7	2.38***	0.73***
Error	21	0.09	0.12

*** significant level at 0.1% level

Table 2. 2. Germination percentages of mature seeds of *P.kawakamii* and *P.tomentosa* treated with alcohol and sodium hypochlorite

Spp	Un-treated	Alcohol	time of hypochlorite soaked					Alcohol + hypo-chlorite
			5 min	15 min	30 min	1 hr	2 hr	
kaw	13c	8c	22b	29b	24b	27b	28b	56a
tom	57d	66cd	75b	72bc	78ab	73bc	72bc	85a

Means separation within species, Tukey's test, 5 % level
Spp: species, Kaw: *P.kawakamii*, tom: *P.tomentosa*

3.2 Effect of water and MS medium with or without agar on the germination of mature seeds of *Paulownia*

Germination of surface sterilized seeds was significantly better than that of untreated seeds (Tables 2. 3 and 2.4). Germination of the untreated seeds varied with species. *P.tomentosa* had the highest at 59 %, while the others were lower *P.fortunei* 28.5 %, *P.taiwaniana* 22.7 % and *P.kawakamii* 12.8 %. Germination of sterilized seeds in water was better than in MS medium for all four species (Table 2. 4). Water with agar gave a better germination percentage than water without agar. *P.fortunei* and *P.taiwaniana* gave very low germination percentage in MS medium. Germination of *P.kawakamii* was better in liquid MS medium than in MS medium solidified with agar while for *P.tomentosa* it was the reverse.

Table 2. 3. Anova of germination percentages of *Paulownia* seeds as effected by germinating media and physical state of the medium

Source of variances	Df	Mean squares for			
		fortunei	*kawakamii*	*tomentosa*	*taiwaniana*
Replication	3	0.10***	0.30***	0.50*	0.66*
Treatments	4	56.49***	8.99***	20.88***	49.86***
Control vs treat[1]	1	4.41***	16.45***	0.02	2.71***
Water vs MS	1	193.34***	16.64***	82.20***	187.50***
Liquid vs solid	1	3.96***	2.62***	0.09	5.41**
Water/MS vs liquid/solid	1	10.44***	0.24*	1.21**	3.82***
Error	11	0.01	0.04	0.12	0.18

*, **, ***: significant level at 0.5, 1, 0.1% level respectively
[1]treat: treatments

Table 2. 4. Effect of seed sterilization, germinating media and physical state of medium on germination percentages of *Paulownia* seeds

Species	Untreated seeds in water (control)	Sterilized seeds in			
		Water		MS medium	
		Liquid	Solid	Liquid	Solid
P.fortunei	28.5c	64.8b	74.5a	0.0e	3.0d
P.kawakamii	12.8e	55.8b	65.3a	34.5c	20.5d
P.tomentosa	59.0b	85.0a	89.0a	18.0d	27.0c
P.taiwaniana	22.7c	55.8b	59.0a	2.5d	0.0d

Means separation within species, Tukey's test, 5 % level.

3.3 Effect of chemical composition of germination medium on seed germination of *P.tomentosa*

Different fractions of MS medium affected the germination percentages of *P.tomentosa* seeds (Tables 2. 5 and 2.6). Seed germination on MS medium + 3 % sucrose were only 22 % as compared to the 81.8 % germination achieved in sterile water. When sucrose was omitted the germination percentage increased to 51.8 %, which did not differ from ½ MS medium or from the macronutrient component of MS medium. In 3 % sucrose 68.8 % of the seeds germinated. When the various macronutrient components of MS medium were tested all except $CaCl_2$ and $MgSO_4$ reduced germination. The micronutrient content of the MS medium did not inhibit germination of *P.tomentosa* seeds.

Table 2. 5. Anova of germination of *P.tomentosa* seeds as affected by germinating media

Source of variances	Df	Mean squares
Replication	3	0.01
Treatments	12	2.63***
Error	35	0.03

*** significant level at 0.1% level

Table 2. 6. Germination percentages of *P.tomentosa* in different fractions of MS medium

Chemical solution	concentration (mM)	Germination %
Sterile water	-	81.8ab
NH_4NO_3	20.600	71.3d
KNO_3	18.800	55.3e
$CaCl_2 \cdot 2H_2O$	3.000	84.3a
$MgSO_4 \cdot 7H_2O$	1.500	80.3bc
KH_2PO_4	1.250	76.8c
$Na_2EDTA + FeSO_4 \cdot 7H_2O$	0.200	76.0c
Sucrose	166.667	68.8d
Micronutrients	0.2361	78.5bc
Macronutrients	45.150	50.7e
½ MS	22.575	54.8e
Full MS	45.586	51.8e
MS + 3 % sucrose	212.253	22.0f

Means separation by Tukey's test, 5 % level.

3.4 Germination of immature seeds.

Sterilized seeds of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* gave higher germination percentages than seeds from sterilized capsules (Tables 2. 7 and 2.8). Alcohol plus hypochlorite thus improved the germination of immature seeds. Germination was better in water than in MS medium for seeds from sterilized capsules of *P.fortunei* and *P.kawakamii* but for *P.taiwaniana* MS medium was better than water (Table 2. 9). When seeds were sterilized, germination was better in water than in MS medium for

P.fortunei and *P.taiwaniana* but for *P.kawakamii* germination did not differ between water and MS medium (Table 2. 10). For *P.fortunei* germination of sterilized seeds was better in medium solidified with agar (Table 2. 11) but for the other two species germination was not affected by the physical state of the medium (liquid or solidified with agar).

Table 2. 7. Anova of germination percentages of *Paulownia* seeds as affected by sterilization procedure, germinating media and physical state of medium

Source of variances	Df	Mean squares for		
		fortunei	*kawakamii*	*taiwaniana*
Replication	3	0.09	0.27	0.04
Treatments	6	4.99***	6.91***	5.01***
Capsule vs sterile seed	1	18.04***	32.94***	25.92***
Capsule water vs MS	1	9.51***	7.22***	2.11***
Capsule MS_l vs MS_s[1]	1	0.92*	0.56	0.39
Sterile seed water vs MS	1	0.79*	0.52	1.20**
Sterile seed liq vs solid	1	0.65*	0.00	0.40
Interaction (Water/MS vs liquid/solid)	1	0.02	0.23	0.01
Error	18	0.11	0.30	0.10

[1] MS_l, MS_s: liquid MS and solid MS medium
*, **, ***: significant at 5, 1, 0.1% level respectively

Table 2. 8. Effect of sterilized procedure on germination percentages of Paulownia seeds

Species	Capsule sterilized	Seeds sterilized
P.fortunei	20.9b	50.6a
P.kawakamii	13.9b	43.6a
P.taiwaniana	30.8b	77.2a

Means separation within species, Tukey's test, 5 % level.

Table 2. 9. Effect of germinating media on germination percentages of seeds retrieved from sterilized capsules of *Paulownia* species

Species	Water	MS medium
P.fortunei	42.3a	10.2b
P.kawakamii	22.5a	5.3b
P.taiwaniana	20.8b	40.8a

Means separation within species, Tukey's test, 5 % level.

Table 2. 10. Effect of germinating media on germination percentages of sterilized seeds of *Paulownia*

Species	Water	MS medium
P.fortunei	56.0a	45.1b
P.taiwaniana	81.9a	72.5b

Means separation within species, Tukey's test, 5 % level.

Table 2. 11. Effect of the physical state of the germinating medium on the germination percentages of sterilized seeds of *Paulownia fortunei*

Species	Water	MS medium
P.fortunei	45.6b	55.5a

Means separation by Tukey's test, 5 % level.

4. DISCUSSION

Alcohol apparently acts only as disinfectant and surfacant (Hu and Wang, 1983). There is no evidence of a stimulative effect as obtained when grass seeds were soaked for 3 days in 3 % alcohol solution (Taylorson & Hendricks, 1979). Improved germination achieved with alcohol and hypochlorite is probably due to their antifungi and antibacteria action (Donald, 1987) but also possibly due to oxidation of inhibitory compounds, increased permeability of the seed coats to oxygen (Frank & Larson, 1970; Major & Wright, 1974; Hsiao 1979 a and b) or a substitute of the light requirement of the seeds (Emal & Conard, 1972). It is also possible that pretreatment with alcohol and Tween 20 enhanced absorption of sodium hypochlorite and therefore increasing the effects mentioned above.

Germination was also better in water solidified with agar. According to Orphanos and Heddecker (1968) seed germination of *Phaseolus vulgaris* can be inhibited by excess water uptake. This may also be the case with *Paulownia*.

MS medium plus 3 % sucrose inhibited germination of sterilized mature seeds of *Paulownia* species. This may be caused by too high a salt concentration or the presence of certain compounds or chemicals (Table 2. 6). NH_4NO_3 and KH_2PO_4

reduced germination percentage of *Oryza sativa* seeds (Mikkelsen & Sinah, 1961). While KNO_3 stimulated germination in many seeds, concentrations above 20 mM inhibited germination of *Epilobium montanum* seeds (Mayer & Polijakoff-Mayber, 1982). High osmotic potential caused by sucrose could also inhibit seed germination (Junttila, 1976).

Germination of immature seeds of *P.kawakamii* sterilized with alcohol plus sodium hypochlorite was not inhibited by MS medium but inhibition did occur for the other two species. However, the inhibition was less than for mature seeds. This could be due to the nutrient requirement of immature seeds. MS medium was used to culture immature embryos of many species to achieve organogenesis and embryogenesis (Ammirato, 1983; Flick *et al.*, 1983). However, MS medium inhibited the germination of immature seeds of *P.fortunei* and *P.kawakamii* for disinfected capsules but stimulated germination of similarly treated *P.taiwaniana* seeds. The reason for this difference in behavior is not clear.

5. REFERENCES

Abdul-Baki, A.A., 1974. Pitfalls in using sodium hypochlorite as a seed disinfectant in ^{14}C incorporation studies. Plant Physiol. 53:768-771

Ammirato, P.V., 1983. Embryogenesis. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture Vol.1: Techniques for propagation and breeding. (pp. 82-123) Macmillan Publishers Co., New York

Borthwick, H.A., Toole, E.H. and Toole, V.K., 1964. Phytochrome control of *Paulownia* seed germination. Isr. J. Bot. 13:122-133

Donald, D.G.M., 1987. Studies on the viability of *Paulownia* seed. Proceedings: Asian Network of Biological Sciences and National Development Research Center, Canada. pp.76-83

Emal, J.G. and Conard, E.C., 1972. Seed dormancy and germination in Indiangrass as affected by light, chilling, and certain chemical treatments. Agronomy J. 65:383-385

Fieldhouse, D.J. and Sasser, M., 1975. Stimulation of pepper seed germination by sodium hypochlorite treatment. HortSci. 10(6):622

Flick, C.E., Evans, D.A. and Sharp, W.R., 1983. Organogenesis. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture Vol. 1: Techniques for propagation and breeding. (pp. 13-81) Macmillan Publishers Co., New York

Frank, A.B. and Larson, K.L., 1970. Influence of oxygen, sodium hypochlorite, and dehulling on germination of green needlegrass seed (*Stipa viridula* Trin). Crop Sci. 10:679-682

Grubisic, D., Konjevic, R. and Neskovic, M., 1988. The effect of some growth regulators on light-induced germination of *Paulownia tomentosa* seeds. Physiol. Plant. 72:525-528

Grubisic, D., Neskovic, M. and Konjevic, R., 1985. Changes in light sensitivity of *Paulownia tomentosa* and *P.fortunei* seeds. Plant Sci. 39:13-16

Hu, V.Y. and Wang, P.J., 1983. Meristem, shoot tip and bud cultures. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture Vol. 1: Techniques for propagation and breeding. (pp. 177-227) Macmillan Publishers Co., New York

Hsiao, A.I., 1979a. The effect of sodium hypochlorite and gibberellic acid on seed dormancy and germination of wild oats (*Avena fatua*). Can. J. Bot. 57:1729-1734

Hsiao, A.I., 1979b. The effect of sodium hypochlorite, gibberellic acid, and light on seed dormancy and germination of wild buckwheat (*Polynomum convovulus*) and cow cockle (*Saponaria vaccaria*). Can. J. Bot. 57:1735-1739

Junttila, O., 1976. Germination inhibitors in fruit extracts of red beet (*Beta vulgaris* cv. Rubra). J. Exp. Bot. 27(99) :827-836

Major, R.L. and Wright, L.N., 1974. Seed dormancy characteristics of Sideoats gramagrass, *Bouteloua curtipendula* (Michx.) Torr. Crop Sci. 37-40

Mayer, A.M. and Polijakoff-Mayber, A., 1982. The germination of seeds. 3rd Ed. (pp. 73-73) Pergamon Press

Mikkelsen, D.S. and Sinah, M.N., 1961. Germination inhibition in *Oryza sativa* and control by preplanting soaking treatments. Crop Sci. 1:332-335

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479

Orphanos, P.I. and Heddecker, W., 1968. On the nature of the soaking injury of *Phaseolus vulgaris* seeds. J. Exp. Bot. 19:770-784

SAS Institute Inc., 1982. SAS user's guide: Statistics, 1982 Ed. SAS Institute Inc. Cary. NC., USA

Snedecor, G.W. and Cochran, W.G., 1980. Statistical methods. 17th ed. (507 pp.) The Iowa State Univ. Press, USA

Taylorson, R.B. and Hendricks, S.B., 1979. Overcoming dormancy in seeds with ethanol and other anesthetics. Planta 145:507-510

Toole, E.H., Toole, V.K. and Borthwick, H.A., 1958. Action of light on germination of seeds of *Paulownia tomentosa* Pro. Plant. Phys. Meetings of AIBS 33 (suppl.) xxii

Wang, T.D. and Hong, F.W., 1979. A study of seed germination and seedling growth of the genus *Paulownia* in Taiwan. Tech. Bull., Exp. Forest, National Taiwan Univ. 123:41-77

CHAPTER 3

IN VITRO CULTURE FOR FOUR *PAULOWNIA* SPECIES

Key words: gelling agents, organogenesis, *Paulownia* species, tissue culture

ABSTRACT

Organogenesis in explants of *Paulownia fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* was studied. Cotyledon, hypocotyl, shoot tip, nodal, internodal and leaf explants were tested. Explants were cultured on MS medium supplemented with different combinations of NAA and BA. All explant types of *P.tomentosa* and *P.taiwaniana* and cotyledon and hypocotyl explants of *P.fortunei* and *P.kawakamii* formed adventitious buds within the concentration range of 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA. Hypocotyl explants produced more adventitious buds than the other explants, while leaf explants produced the least. Adventitious bud and root formation on the other four explants for *P.fortunei* and *P.kawakamii* occurred over a wide range of NAA + BA combinations. Adventitious bud formation of internodal explants of *P.tomentosa* was inhibited by Biolab agar but not by Difco agar. Biolab agar induced more callus growth than Difco agar for all four species. *P.fortunei* and *P.tomentosa* formed callus more readily than the other two species.

ABBREVIATIONS USED

BA = 6-benzylaminopurine; IAA = indole acetic acid; NAA = α-naphthaleneacetic acid

1. INTRODUCTION

Organogenesis from different juvenile explants of *P.elongata*, *P.elongata* x *tomentosa*, *P.fortunei*, *P.taiwaniana* and *P.tomentosa* have been reported (Fan & Hu, 1976; Yang, 1982; Marcotrigiano & Stimart, 1983; Huang, 1985; Ho *et al.*, 1988; Chang & Donald, 1992). Adventitious buds formed readily on different explants of *Paulownia*. Growth regulators used by researchers were combination of IAA and kinetin or NAA and BA. Adventitious buds formed less readily when combination of IAA and kinetin were used in place of NAA and BA combination. This study reports on the organogenetic potential of different explants from juvenile tissues of four *Paulownia* species.

2. MATERIAL AND METHODS

2.1 Establishment of seedling and shoot tip subculture

Seeds of *P.fortunei*, *P.kawakamii*, *P.taiwaniana* and *P.tomentosa* were surface sterilized for 1 min in 70% alcohol then 15 min in commercial bleach (NaOCl) plus a drop of Tween 20 per 100 ml followed by 3 rinses with sterile water. Seeds were placed in 220 ml glass jars containing 30 ml MS basal medium (Murashige & Skoog, 1962) supplemented with 3 % sucrose and 0.2 % gelrite (Kelco, Merk Chemical Co.). After 3 to 4 weeks, seedlings were 5-8 mm tall and had 2 cotyledons and 2 small

leaves. Seedling shoot tips ca. 3 mm long were subcultured onto MS medium solidified with 0.7 % Difco (Difco Lab.) or Biolab agar (Biolab Diagnostics Ltd). When the plantlets were 5 cm tall shoot tips were subcultured. This was done every 1½ months in order to have a regular supply of plant material for experimental usage.

2.2 Organogenesis using different explants on gelrite

Explants of four *Paulownia* species consisted of: cotyledons cut into two equal portions through the middle; hypocotyls, shoot tips, nodes and internodes were cut into 2-3 mm long sections; and 3 mm square leaf fragments cut from the central part of the leaves. Cotyledon and hypocotyl explants were prepared from 3-4 week old *in vitro* grown seedlings. The other explants were prepared from *in vitro* grown shootlets. Only shootlets which were subcultured twice were used for shoot tip, nodal, internodal, and leaf explants. Three explants were used per test tube which contained 5 ml media. The medium used was MS medium solidified with 0.2% gelrite and supplemented with NAA (0.5,1.0 or 2.5 mg l^{-1}) and BA (0.5, 1.0,2.5, or 5.0 mg l^{-1}). Test tubes were capped with 2 layers of aluminum foil. Treatments were replicated four times. The number of adventitious buds and roots per explant was recorded after 4 weeks for *P.kawakamii* and 6 weeks for the other three species.

2.3 Organogenesis of internodal explants on agar

Internodal sections of four *Paulownia* species were placed into test tubes containing 5 ml MS medium, supplemented with 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA and solidified with 0.6 % and 0.8 % of either Difco or Biolab agar. Three internodal explants were used per treatment and treatments were replicated five times. After 5 weeks in culture,

the number of adventitious buds per explant was recorded and fresh weight of explants determined.

2.4 Histological study

During the early stages of organogenesis internodal explants of the four species were selected and fixed in formalin-acetic acid-alcohol (FAA), dehydrated in tertiary butyl alcohol series (Johanson, 1940) and embedded in paraffin wax. Sections 10 μm thick were cut and stained with safranin-fast green (Sass, 1958).

In this series of experiments the pH of the media was adjusted to 5.7 by using NaOH to maintain all the salts in the soluble form before autoclaving at 121^{o}C for 15 min (Murashige & Skoog, 1962). Cultures were kept in a culture room at 25 ^{o}C under fluorescent light of 30 $\mu Em^{-2}s^{-1}$ with a 16 hour photoperiod.

3. RESULTS AND DISCUSSION

3.1 Organogenesis in explants cultured on gelrite solidified media

Callus growth occurred just inside the epidermis layer of the explants. Adventitious buds were visible after two weeks and they increased in number until the fourth week in culture (Fig. 3.1). Sections prepared of explants after two weeks in culture revealed that adventitious buds originated from the callus tissue (Fig. 3.2-a). Adventitious buds also formed in the subepidermal parenchyma cells of internodal explants of *P.tomentosa* (Fig. 3.2-b). Many meristemoids with vascular bundles formed in this tissue. Thus both direct and indirect organogenesis occurred in *P.tomentosa* explants.

Direct organogenesis refers to meristemoid formation without a callus interphase as compared to meristemoid formation from explant derived callus tissue (Hicks, 1980).

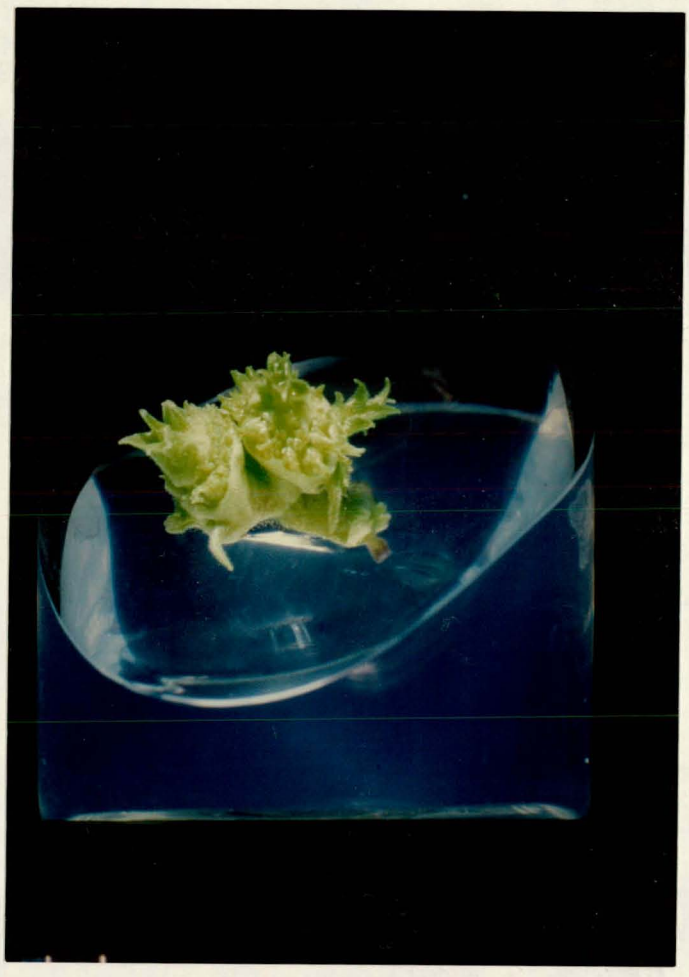


Fig. 3. 1. Adventitious bud formation in callus tissue of internodal explants of *P.kawakamii*. Callus developed on the cut surfaces. Bar represents 1 cm.

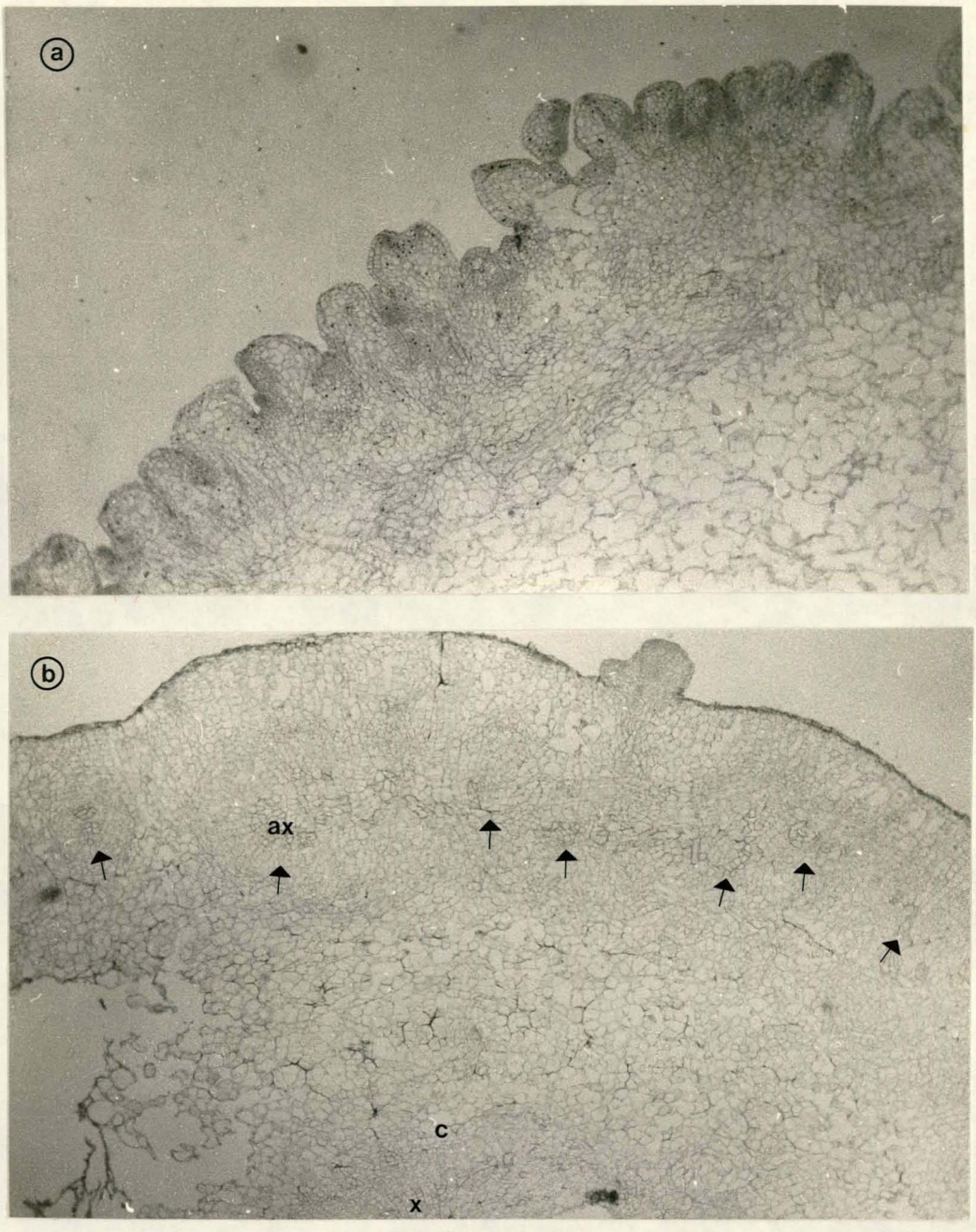


Fig. 3. 2. (a) Longitudinal section of internodal explants of *P.fortunei*, adventitious buds formed from callus growth on the cut surface. (b) cross section of internodal explants of *P.tomentosa*, vascular bundles of adventitious buds formed from cortex tissue (arrows). x: xylem, c: cambium, ax: xylem of adventitious bud, bar represents 200 μm.

Cotyledon and hypocotyl explants of all four species formed adventitious buds only in the narrow concentration range of NAA + BA (0.5 + 5.0 mg l^{-1}). The number of adventitious buds per explant on medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA varied from 13 to 46 (Table 3. 1). Shoot tip, nodal, internodal and leaf explants of *P.tomentosa* and *P.taiwaniana* reacted similarly, forming adventitious buds predominantly in the range of 0.5 mg l^{-1} NAA + 5 mg l^{-1} BA. Very few adventitious buds formed on the explants which where cultured in other combinations of NAA + BA.

Table 3. 1. Adventitious bud formation on different explants of four *Paulownia* species cultured *in vitro*[1].

Paulownia species	Adventitious buds/explant					
	Coty	Hyp	Sht tip	Node	Internode	Leaf
fortunei	15±2.3	46±2.5	20±3.8	17±1.6	19±3.1	10±2.3
kawakamii	13±2.6	29±1.6	19±2.2	24±3.1	31±3.8	4±1.0
tomentosa	13±2.2	44±2.0	34±10.9	9±3.4	2±1.0	2±0.6
taiwaniana	26±3.0	23±3.8	17±3.5	9±0.5	32±7.5	15±1.1

[1]Medium is MS medium supplemented with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} BA.
Coty: cotyledon, Hyp: hypocotyl, Sht tip: shoot tip

The combination of NAA and BA appear more effective than IAA and kinetin (Fan & Hu, 1976; Marcotrigiana & Stimart, 1983) in inducing adventitious bud formation. NAA and BA were more effective in controlling the growth and development of a wide range of gymnosperm species than IAA and IBA (Durzan, 1983). IAA which is known to break down *in vitro*, is not as effective an auxin as 2,4-D or NAA in shoot formation of Solanaceous species (Flick *et al.*, 1983). Ho *et al.* (1988) found BA superior to

kinetin in adventitious bud formation from shoot tip explants of *P.taiwaniana*. The same result was obtained when shoot tip explants of *P.tomentosa* were cultured on BA (Marcotrigiano & Stimart, 1983). The narrow range of NAA + BA found effective in this study could be due to the higher concentration of NAA used. Ho *et al.* (1988) reported that when 0.2 mg l^{-1} NAA and 3 or 5 mg l^{-1} BA were used many adventitious buds formed on nodal, internodal and leaf explants of *P.taiwaniana*. Adventitious bud formation was however suppressed by 1 mg l^{-1} NAA. IBA from 0.1 to 10 mg l^{-1} and combined with a BA concentration of 1 mg l^{-1} decreased the number of adventitious buds per explant in shoot tip subculture of *P.tomentosa* (Marcotrigiano & Stimart, 1983).

For *P.fortunei* and *P.kawakamii* adventitious bud formation on shoot tip, nodal, internodal and leaf explants formed in a wide range of NAA:BA concentrations. The anova of the number of adventitious buds as affected by different concentration of NAA and BA is presented in Table 3. 2. The number of adventitious buds formed per explant did not differ for shoot tip, nodal and internodal explants for both species (Table 3. 3). Leaf explants of *P.kawakamii* formed fewer adventitious buds than the other explants whereas for *P.fortunei* leaf explants differed only from shoot tip explants. Increasing the concentration of NAA from 0.5 to 2.5 mg l^{-1} reduced the number of adventitious buds in nodal and internodal explants but not in shoot tip and leaf explants (Table 3. 4). Increasing concentration of BA from 0.5 to 5.0 mg l^{-1} caused more adventitious buds to form in *P.fortunei* but for *P.kawakamii* there appeared to be no additional stimulation of adventitious buds above 1.0 mg l^{-1} (Table 3. 5). Bud and root formation was correlated negatively. Increasing NAA concentration did not affect the number of roots formed in nodal and internodal explants of both species and leaf explants of *P.kawakamii*. Shoot tip explants reacted positively to an increase in NAA concentration and roots formed more readily in the

case of *P.fortunei* than *P.kawakamii*. In the case of leaf explants of *P.fortunei* no differences in root formation occurred when NAA increased above 1.0 mg l^{-1} (Table 3. 6). Explants of *P.kawakamii* formed fewer roots than that of *P.fortunei* (Table 3. 7). Increasing BA concentrations suppressed root formation in explants of *P.fortunei* but not in explants of *P.kawakamii*. Nodal and internodal explants formed few roots and were not affected by BA concentration. Root formation in shoot tip and leaf explants was increasingly inhibited by increasing BA concentrations (Table 3. 8).

Organogenesis of *P.fortunei* and *P.kawakamii* depended on the NAA + BA concentrations and explant types. Genotype affects the organogenetic response. Species, cultivar and plant-dependent regeneration has been reported in many species such as *Cucumis* (Wehner & Locy, 1981), *Hordeum* (Hanzel *et al.*, 1985), *Lycopersicon* (Zelcer *et al.*, 1984), *Medicago* (Brown & Atanassov, 1985). The reason why shoot tip explants formed adventitious buds more readily is possibly due to more mitotically active cells (Murashige, 1979). Different tissues or organs of a plant may differ in their organogenetic potential. Differences in the phenotypic physiological condition of the cells in the original explant (Bajaj & Bopp, 1972) or differences in their requirement for cytokinin or auxin (Blumenfeld & Gazit , 1971; Wu & Li, 1971; Meins & Lutz, 1979; Meins, 1982) or differences in ploidy (Murashige & Nakano, 1967; Shimada & Tabata, 1967; Marchetti *et al.*, 1975) may all affect the response of the tissue in culture.

Table 3. 2. Anova of number of adventitious buds and roots as affected by NAA and BA, explant type and species *(P.fortunei* and *P.kawakamii*).

Source of Variation	Df	Mean Squares for	
		Adventitious buds	Roots
Replication	3	102	5.7
Spp[1]	1	52	93.3*
Exp[2]	3	2150*	25.1*
NAA	2	1998*	20.2*
BA	3	1606*	37.4*
SppxExp	3	1064*	12.7*
NAAxBA	6	145	1.9
NAAxSpp	2	17	10.0
BAxSpp	3	313*	17.7*
NAAxExp	6	346*	17.9*
BAxExp	9	128	9.7*
NAAxBAxSpp	6	125	0.9
NAAxBAxExp	18	53	3.2
NAAxSppxExp	6	127	9.5*
BAxSppxExp	9	173	5.5
NAAxBAxSppxExp	18	89	2.2
Error	279	78	2.6

*: significant at 1 % level
[1]Spp: species, [2]Exp: explant type

Table 3. 3. Interaction between explant types and *Paulownia* species on adventitious bud formation (buds/explant)

Species	Shoot tip	Node	Internode	Leaf
P.fortunei	15a	11ab	9ab	5bc
P.kawakamii	9ab	17a	17a	1c

Means separation by Tukey's test, 1 % level

Table 3. 4. Interaction between NAA concentration and explant type on adventitious bud formation of *Paulownia* (buds/explant)

NAA mg l^{-1}	Shoot tip	Node	Internode	Leaf
0.5	16abc	18ab	21a	4ef
1.0	14abcde	14abcd	11bcdef	4ef
2.5	9cdef	9cdef	7def	3f

Means separation by Tukey's test, 1 % level

Table 3. 5. Interaction between BA concentration and *Paulownia* species on adventitious bud formation (buds/explant)

BA mg l^{-1}	*P.fortunei*	*P.kawakamii*
0.5	5c	7bc
1.0	8bc	11abc
2.5	10abc	13abc
5.0	18a	14ab

Means separation by Tukey's test, 1 % level

Table 3. 6. Interaction between NAA concentration and explant type and species on adventitious root formation (roots/explant)

NAA mg l^{-1}	Shoot tip		Node		Internode		Leaf	
	fot	kaw	fot	kaw	fot	kaw	fot	kaw
0.5	1.6b	0.2c	0.3c	0.1c	0.0c	0.2c	0.8c	0.2c
1.0	1.9b	0.7c	0.4c	0.2c	0.1c	0.2c	2.1b	0.2c
2.5	6.2a	1.2b	0.5c	0.1c	0.2c	0.1c	1.4b	0.4c

Means separation by Tukey's test, 1 % level
fot: *P.fortunei*, kaw: *P.kawakamii*

Table 3. 7. Interaction between NAA concentration and *Paulownia* species on adventitious root formation (roots/explant)

BA mg l^{-1}	*P.fortunei*	*P.kawakamii*
0.5	2.5a	0.5bc
1.0	1.9ab	0.4c
2.5	0.5bc	0.2c
5.0	0.3c	0.1c

Means separation by Tukey's test, 1 % level

Table 3. 8. Interaction between BA concentration and explant type on adventitious root formation (roots/explant)

BA mg l^{-1}	Shoot tip	Node	Internode	Leaf
0.5	3.6a	0.6c	0.2c	1.6abc
1.0	2.9ab	0.4c	0.3c	1.2bc
2.5	0.9bc	0.1c	0.1c	0.4c
5.0	0.5c	0.1c	0.0c	0.2c

Means separation by Tukey's test, 1 % level

3.2 Effect of agar brand on adventitious bud formation

Extensive vitrification of shoots and excessive callus growth occurred when gelrite was used as gelling agent. Even in hormone free medium vitrification occurred. The degree of vitrification was species dependent. In *P.tomentosa* vitrification occurred after the first subculture on gelrite solidified medium, for *P.fortunei* after the second subculture in the distal shoot sections and for *P.kawakamii* and *P.taiwaniana* after the third subculture at the shoot tips. Vitrification did not occur on 0.7 % Difco or Biolab agar solidified media even after many subcultures. The anova of number of adventitious buds and fresh weight as affected on concentration and species is presented in Table 3. 9. Agar brand had no effect on adventitious bud formation of internodal explants of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* (Table 3. 10). However, Biolab agar inhibited adventitious bud formation in internodal explants of *P.tomentosa*. Internodal explants of *P.fortunei* formed adventitious buds more readily than the other species. Biolab agar produced prolific callus growth and therefore explants had a greater fresh weight (Table 3. 11). *P.kawakamii* and *P.taiwaniana* formed callus less readily as revealed by the lower fresh weight (Table 3. 12). Different brands of agar

contain various impurities and thus affect the chemical and hysical characteristics of a culture medium (Debergh, 1983). Pochet *et al.* (1991) pointed out the bud formation of *Thuja plicata* explants decreased when an agar brand containing higher sulfate was used as gelling agent..

Table 3. 9. Anova of number of adventitious buds and fresh weight as affected by agar brand and concentration and species

Source of Variation	Df	Mean squares for	
		Adventitious buds	Fresh weight
Replication	4	85	58660
Agar	1	401	3772764*
Agrconc[1]	1	154	710080
Spp	3	1320*	1206552*
AgarxAgrconc	1	31	160116
AgarxSpp	3	617*	431753
AgrconcxSpp	3	182	272229
AgarxagrconxSpp	3	218	445219
Error	60	60	170715

[1]medium is MS medium supplemented with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} BA.
*: significant at 1 % level, [1]Agrconc: concentration of agar

Table 3. 10. Interaction between agar brand and *Paulownia* species on adventitious bud formation (buds/explant)

Agar brand	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
Difco	31a	22b	22b	20b
Biolab	33a	16b	2c	26ab

Means separation by Tukey's test, 1 % level

Table 3. 11. Effect of agar brand on fresh weight (mg) per internodal explant between agar brand

Agar brand	Fresh weight (mg)
Difco	392b
Biolab	609a

Means separation by Tukey's test, 1 % level

Table 3. 12. Effect of agar brand on fresh weight (mg) per internodal explant among four *Paulownia* species

	fortunei	*kawakamii*	*tomentosa*	*taiwaniana*
Fresh weight	621a	386b	594a	406b

Means separation by Tukey's test, 1 % level

4. REFERENCES

Bajaj, Y.P.S. and Bopp, M.Z., 1972. Growth and organ formation in *Sinapis alba* tissue cultures. Z. Pflanzenphysiol. 66:378-381

Blumenfeld, A. and Gazit, S., 1971. Growth of avocado fruit callus and its relation to exogenous and endogenous cytokinins. Physiol. Plant. 25:369-371

Brown, D.C.W. and Atanassov, A.I., 1985. Role of genetic background in somatic embryogenesis in *Medicago*. Plant Cell, Tissue & Organ Cult. 4:111-122

Chang, S.H. and Donald, D.G.M., 1992. *In vitro* organogenesis and plantlet formation of *Paulownia elongata*. South Africa For. J. in press

Debergh, P.C., 1983. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plnat. 59:270-276

Durzan, D.J., 1983. Cell and tissue culture in forest industry. In: Bonga, J.M. and Durzan D.J. (Eds.) Tissue culture in forestry. 2nd printing (pp. 36-71) Martinus Nijhoff Publishers, The Netherlands

Fan, M.L. and Hu, T.W., 1976. Plantlets from *Paulownia* tissue cultures. Bull. of Taiwan For. Res. Inst. No. 286:1-6

Flick, C.E., Evans, D.A. and Sharp, W.R., 1983. Organogenesis. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell

culture Vol.1: Techniques for porpagation and breeding. (pp. 13-81) Macmillan Publishing Co., New York

Hanzel, J.J., Miller, J.P., Brinkmann, M.A. and Fendos, E., 1985. Genotype and media effects on callus formation and regeneration in barley. Crop Sci. 25:27-37

Hicks, G.S., 1980. Patterns of organ development in plant tissue culture and the problem of organ determination. Bot. Rev. 46:1-23

Ho, C.K., Chang, S.H. and Yang, J.C., 1988. Tissue culture of *Paulownia taiwaniana* from juvenile tissues. Sym. of Tree Improvement & Tissue Cult. in Taiwan. (pp 97-113) Exp. For. of Nat. Taiwan Univ.

Huang, C., 1985. *In vitro* culture and plantlet regeneration from immature embryo of *Paulownia elongata* x *tomentosa*. Plant Physiol. Commun. 1:39 (in Chinese) In: Chen, Z., Evans, D.A., Sharp, W.R., Ammirato, P.V. and Sondahl, M.R. (Eds.) 1990. Handbook of plant cell culture. Vol.6 (p. 39) McGraw-Hill Publishing Company, New York

Johanson, D.A., 1940. Plant microtechnique. McGraw-Hiü. New York. In: Sass, J.E. (Ed.) 1958. Botanical microtechnique. 3rd ed. The Iowa State Univ. Press

Marchetti, S., Kiss, Z.R. and Brunori, A., 1975. Time course of polyploidization in calli derived from stem and pith explants of *Nicotiana tabacum* studied on isolated nuclei. Z. Pflanzenphysiol. 78:307-313

Marcotrigiano, M. and Stimart, D.P., 1983. *In vitro* organogenesis and shoot proliferation of *Paulownia tomentosa* Steud. (Empress tree). Plant. Sci. Lett. 31:303-310

Meins, F.Jr., 1982. The nature of the cellular, heritable, change in cytokinin habituation. In: Earle, E. and Demarly, Y. (Eds.) Variability in plants regenerated from tissue culture. (pp. 202-210) Praeger, New York

Meins, F.Jr. and Lutz, J., 1979. Tissue specific variation in the cytokinin habituation of cultured tobacco cells. Differentiation 15:1-6

Murashige, T., 1979. Principles of rapid propagation. In: Hughes, K.W. Henke, R. and Constantin, M. (Eds.) Propagation of higher plants through tissue culture. USDOE, Washington, DC

Murashige, T. and Nakano, R., 1967. Chromosome complement as a determination of morphogenetic potential of tobacco cells. Am. J. Bot. 54:963-970

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479

Marcotrigiano, M. and Stimart, D.P., 1983. *In vitro* organogenesis and shoot proliferation of *Paulownia tomentosa* Steud. (Empress tree). Plant. Sci. Lett. 31:303-310

Marcotrigiano, M. and Stimart, D.P., 1983. *In vitro* organogenesis and shoot proliferation of *Paulownia tomentosa* Steud. (Empress tree). Plant. Sci. Lett. 31:303-310

Pochet, B., Scoman, V., Mestdagh, M.M., Moreau, B. and Ardre, P., 1991. Influence of agar gel properties on the *in vitro* micropropagation of different clones of *Thuja plicata*. Plant cell Rep. 10:406-409

Sass, J.E., 1958. Botanical microtechnique. 3rd ed. The Iowa State Univ. Press

Schimada, T. and Tabata, M. 1967. Chromesome numbers in cultured pith tissue of tobacco. Japan J. Genet. 42:195-201

Wehner, T.C. and Locy, R.D., 1981. *In vitro* adventitious shoot and root formation of cultivars and lines of *Cucumis sativus* L. HortSci. 16:759-760

Wu, L. and Li, H.W., 1971. Induction of callus tissues initiation from different somatic organs of rice plant by various concentration of 2,4-dichlorophenoxy acetic acid. Cytologia 36:411

Yang, N., 1982. Tissue culture and organogenesis of some woody plants *in vitro*. Plant Physiol. Commun. 4:23-27 (in Chinese) In: Chen, Z., Evans, D.A., Sharp, W.R., Ammirato, P.V. and Sondahl, M.R. (Eds.) 1990. Handbook of plant cell culture Vol.6: Perennial crops. (p. 39) McGraw-Hill Publishing Company, New York

Zelcer, A., Soferman, O. and Izhar, S., 1984. An *in vitro* screening for tomato genotypes exhibiting efficient shoot regeneration. J. Plant Physiol. 115:211-215

CHAPTER 4

VITRIFICATION IN TISSUE CULTURE OF *PAULOWNIA*

key words: gelling agents, *Paulownia* species, tissue culture, vitrification

ABSTRACT

Vitrification of adventitious buds induced from internodal explants of *Paulownia fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* was studied. Different concentrations of agar, cobalt, inositol, MS macrosalts, growth regulators and different light intensities did not effectively eliminate vitrification, but increasing light intensity increased adventitious bud formation. *P.taiwaniana* formed fewer vitrified shoots than *P.fortunei* and *P.kawakamii*. Gelrite formed more adventitious buds but caused more vitrification than agar. The morphological characteristics and growth of normal and vitrified shoots were studied when subcultured on different gelling agents and concentrations. Vitrified shoots of *P.fortunei* and *P.tomentosa* produced more callus on the cut surfaces of shoots whereas the other two species formed more axillary shoots. Vitrified shoots had a higher growth rate than normal shoots but the ability to form roots did not differ from normal shoots. Increasing the concentration of gelling agents inhibited shoot growth except for *P.kawakamii* which had an inherently slow growth rate on all concentrations of gelling agents tested. Vitrified shoots decreased in water content and reverted to normal shoots when cultured on 0.8 % or higher Difco agar, but not on gelrite nor mixtures of gelrite and agar.

ABBREVIATIONS USED

BA = 6-benzylaminopurine; NAA = α-naphthaleneacetic acid;

1. INTRODUCTION

Vitrification (synonyms: glassiness, translucency, vitrescence, hyperhydric malformations) is a metabolic and morphological derangement affecting herbaceous and woody plants during *in vitro* propagation (Gaspar *et al.*, 1987; Ziv, 1990). In general, stems of vitrified plantlets are broad, thick and translucent, leaves are thick, wrinkled and/or curled, frequently very elongated, and easily broken (Gaspar *et al.*, 1987). In some cases friable callus develop and axillary shoots are induced (Böttcher *et al.*, 1988). Ziv (1990) reviewed the effect of cultural conditions on the vitrification of micropropagated plants. She listed the following conditions that can contribute to vitrification: culture environment (liquid vs solid medium and culture atmosphere), organic and inorganic components (gelling agent, carbohydrates and mineral nutrients), growth regulators (cytokinins and auxins), light and temperature. Debergh (1987) showed that the relative humidity in the container and the water potential of culture medium were the key factors in vitrification.

Vitrification of *in vitro* cultures of *Paulownia* species were first observed by Ho *et al.* (1988). Vitrification occurred in subcultured shoot tips on MS medium (Murashige & Skoog, 1962) solidified with 0.2 % gelrite and in adventitious buds formed on explants growing in MS medium containing growth regulators (Chapter 3). In this study we report on the same factors which affect vitrification.

2. MATERIAL AND METHODS

2.1 Effect of medium composition on vitrification

Vitrification was a general problem in tissue cultures of *Paulownia* when MS medium containing 0.5 mg l^{-1} NAA, 5.0 mg l^{-1} BA and solidified with agar or gelrite was used. A number of media variations were therefore tested in an attempt to overcome this problem. The following variation were attempted: 1. Difco agar concentration increased to 1.0 and 1.2 %. 2. Myo-inositol concentration of MS medium increased to 500 and 1000 mg l^{-1}. 3. Cobalt concentration of MS medium increased to 10^{-6}, 10^{-5} and 10^{-4} M. 4. Sucrose at 1.5 % or 4.5 %. 5. Macronutrients of MS medium decreased to 1/2 and 1/3. 6. NAA + BA decreased to 0.1 mg l^{-1} NAA + 1.0 mg l^{-1} BA or 0.25 mg l^{-1} NAA + 2.5 mg l^{-1} BA. Internodal explants (ca 2-3 mm long) of four *Paulownia* species were used. Each test tube contained 5 ml media and three explants. Explants were excised from *in vitro* grown plantlets which had been subcultured regularly for 1.5 years from shoot tips. Treatments were repeated five times. After 5 weeks in culture, the numbers of adventitious bud formations per explant and percentage of explants which formed vitrified adventitious bud (vitrification percentage) were determined.

The effect of different light intensities on the vitrification was also studied. Culture medium used was MS medium containing 0.5 mg l^{-1} NAA, 5 mg l^{-1} BA and 0.6 or 0.8 % Difco agar or 0.2 % gelrite. Cultures were kept at different light intensities viz. 100, 60, 30 $\mu Em^{-2}s^{-1}$. Explant type and size were as above. Treatments were repeated five times. After 5 weeks in culture the number of adventitious bud formation and vitrification percentage were determined.

2.2 Effect of gelling agent on vitrification

Normal and vitrified shoots (ca 1 cm tall) were cultured on hormone free MS medium solidified with different gelling agents. Normal shoots were cut from plantlets cultured on agar and vitrified shoots from gelrite solidified medium. The gelling agents used were Difco agar (0.6, 0.8, 1.0, and 1.2 %), gelrite (0.2, 0.3 and 0.4 %), and 0.1 % gelrite mixed with 0.3, 0.4 and 0.5 % Difco agar. Each tube contained 5 ml media and one shoot explant. Treatments were repeated 5 times. After 1 month in culture, the fresh weight of the plantlets was determined, and the dry weight obtained after drying for 2 days at 70 ^{o}C. The degree of vitrification was scored: 0 = normal plantlet, 1 = shoot tip or basal parts vitrified; 2 = half of the plantlet vitrified and 3 = whole plantlet vitrified. Callus size on the basal cut surface of the explants was also scored 0, 1, 2, and 3 representing none, small, medium and large calli.

In these series of experiments the pH of the media was adjusted to 5.7 before autoclaving at 121^{o}C for 15 min. Cultures were kept in a culture room at 25 ^{o}C under fluorescent light of 30 $\mu Em^{-2}s^{-1}$ with a 16 hour photoperiod unless otherwise stated.

3. RESULTS AND DISCUSSION

3.1 Effect of medium composition on vitrification

Vitrification was species dependent. *P.fortunei* and *P.tomentosa* generally formed more vitreous buds than the other two species (Table 4.1). Vitreous buds formed randomly. Both vitrified and normal adventitious buds occurred on the same culture which is similar to the phenomena described by Gaspar *et al.* (1987) for apple cultures. Increased concentrations of agar, cobalt and inositol, and decreased concentrations of sugar, MS macrosalts and NAA and BA concentration were reported to reduce the

incidence of vitrification (Debergh *et al.*, 1981; Debergh, 1983, Ziv *et al.*, 1981 and 1983; Leshem, 1983;, Van Arnold & Eriksson, 1984; Bornman & Bogelmann, 1984; Gaspar & Kevers, 1985; Pasqualetto *et al.*, 1986; Gaspar *et al.*, 1987; Orlikowska, 1987; Zimmerman & Cobb, 1989; Phan, 1991; Williams & Taji, 1991). In most of the treatments only a few adventitious buds formed which prevented an objective evaluation of vitrification (Table 4.1). It was however clear that vitrification was more serious on gelrite medium than on agar medium. No treatment gave both a high regeneration rate combined and a low incidence of vitrification. The numbers of adventitious buds formed per internodal explant in this experiment were 1.5 to 4 times lower than that reported in Chapter 3. This appear to be related to the extended period in culture of the explant material. After 1.5 years of serial subcultures of shoot tips, shoots produced a more lignified shoot with a greater stem diameter and thicker dark green leaves. Adventitious bud formation has been reported to decrease as tissues become older (George & Sherrington, 1984).

3.2 Effect of light intensity on vitrification of adventitious buds

Higher light intensity (100 $\mu Es^{-2}m^{-1}$) did not reduce vitrification but enhanced adventitious bud formation (Tables 4.2 and 4.3). George and Sherrington (1984) pointed out that the relationship between shoot proliferation and light intensity was species dependent. *P.taiwaniana* was less prone to vitrification than *P.fortunei* and *P.kawakamii* (Table 4.4). Gelrite induced more vitrification in the latter two species than agar (Table 4.4). Increasing agar concentrations from 0.6 % to 0.8 % reduced vitrification in *P.fortunei* but not for the other two species (Table 4.4). More adventitious buds formed on gelrite as compared to agar solidified media (Table 4.5). Similar results have been reported for many woody plants (MacRae & Van Staden, 1980; McCown & McCown, 1987; Nairn, 1987; Goldfarb *et al.*, 1991).

Table 4.1. The effect of medium composition on adventitious buds per explant and percentages of vitrified cultures. Internodal explants cultured on MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA.

Treatments	*P.fortunei*		*P.kawakamii*		*P.tomentosa*		*P.taiwaniana*	
	ABs[1]±STD	Vit%[2]	ABs±STD	Vit%	ABs±STD	Vit%	ABs±STD	Vit%
Agar 0.6%	8.2±1.4	20	2.1±0.3	20	0 ± 0	-	2.4±0.4	25
0.8%	9.9±1.3	11	5.6±0.8	14	2.4±0.4	33	8.4±1.0	22
1.0%	10.1±1.7	14	5.2±0.6	0	1.3±0.4	0	4.2±0.8	0
Gelrite 0.2%	8.6±2.3	86	4.1±1.0	60	0 ± 0	-	17.3±2.3	50
1/2 MS	21.4±2.6	25	8.6±0.7	11	4.7±0.8	40	7.7±0.6	0
1/3 MS	0.8±0.2	100	4.5±0.8	0	2.7±0.2	75	4.1±0.3	13
Sugar 1.5%	5.4±0.9	80	1.6±0.4	100	0 ± 0	-	3.0±0.4	50
4.5%	5.3±1.0	50	1.9±0.4	0	0 ± 0	-	0 ± 0	-
$CaCl_2$ 10^{-6}	1.3±0.3	100	1.1±0.3	0	0.7±0.2	50	4.1±0.8	0
10^{-5}	0.3±0.1	0	0 ± 0	-	0 ± 0	-	1.9±0.6	0
10^{-4}	0 ± 0	-	0 ± 0	-	0 ± 0	-	0 ± 0	-
Inositol 5%	6.3±1.1	67	1.3±0.5	0	0.6±0.2	0	7.0±1.0	0
10%	2.1±0.4	83	1.3±0.4	0	0.6±0.2	0	6.6±0.4	0

STD: standard deviation
[1]ABs: adventitious buds, [2]Vit%: vitrification %

Table 4.2. Anova of adventitious buds per explant and percentages of cultures vitrified as affected by different light intensities and gelling agent. Internodal explants cultured on MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA.

Source of variance	Df	Adventitious buds/internode	Vitrification
Replication	4	10.1	697
Gel[1]	2	171.0*	10293*
Light	2	367.9*	1155
Spp	2	5.2	17036*
Gelxlight	4	20.3	1952
Gelxspp	4	3.5	4178*
Lightxspp	4	56.5	1647
Gelxlightxspp	8	33.5	1214
Error	89	26.8	1144

[1]Gel: gelling agents, *: significant at 1 % level

Table 4.3. Effect of light intensity on the formation of adventitious buds per internodal explants of Paulownia.

Light intensity	Adventitious buds/internode
100 $\mu Em^{-2}s^{-1}$	8.1a
60 $\mu Em^{-2}s^{-1}$	3.0b
30 $\mu Em^{-2}s^{-1}$	3.0b

Means separation by Tukey's test, 1 % level

Table 4.4. Effect of different gelling agents and concentration on % cultures vitrified in 3 species of *Paulownia*.

Gelling agents	*P.fortunei*	*P.kawakamii*	*P.taiwaniana*
Agar 0.6 %	33.3bc	33.3bc	1.4e
Agar 0.8 %	10.0de	17.8cd	1.3e
Gelrite 0.2 %	88.7a	44.4b	6.6de

Means separation by Tukey's test, 1 % level

Table 4.5. Effect of different gelling agents on the formation of adventitious buds per internodal explants of *Paulownia*.

	Agar 0.6 %	Agar 0.8 %	Gelrite 0.2 %
Adventitious buds per internode	3.1b	4.0b	7.2a

Means separation by Tukey test, 1 % level

3.3 Effect of gelling agents and concentration on the morphogenesis and growth of vitrified and normal shoots

3.3.1 Effect on morphological characteristics

Various gelling agents at different concentrations significantly affected the morphological characteristics such as vitrification, callus growth on the cut surfaces and axillary shoot formation for both normal and vitrified shoots of four *Paulownia* species (Table 4.6). Vitrified shoots reverted almost completely to normal shoots when cultured on media solidified with 0.8 % or more agar (Table 4.7). Increasing gelrite or

gelrite + agar concentration did not reduce the incidence of vitrified shoots. Vitrified shoots of *P.taiwaniana* and *P.kawakamii* reverted to normal shoots more readily than the other two species (Table 4.8). High levels of gelrite decreased vitrification of *Picea abies* and *Prunus amygdalus* cultures (Van Arnold & Eriksson, 1984; Rugini *et al.*, 1987; Williams & Jaji, 1991) but not *Malus domestica* cultures (Pasqualetto *et al.*, 1988). Pasqualetto *et al.* (1988) suggested that the higher incidence of vitrification on gelrite media could be due to the higher levels of K^+ and Mg^{++}. The finding that mixtures of gelrite and agar did not improve vitrification supports the view that high K^+ and Mg^{++} levels may cause the vitrification. Gelrite mixed with agar has reduced vitrification of some woody species (Pasgualetto, *et al.*, 1986; McCown & McCown, 1987; Nairn, 1987) but it failed to reduce vitrification for *Paulownia* species. Irrespective of species and gelling agent, little or no vitrification was observed on normal shoots when single explant was grown in a tube (Tables 4.7 and 8). This may account for the near absence of vitrification as compared to earlier work (Chapter 3) when 20 explants were cultured per jar. High explant density in the jar could cause an increase in water vapor, CO_2 and ethylene content of the culture atmosphere, factors known to enhance vitrification (Ziv, 1990).

Vitrified shoots produced more callus than normal shoots (Table 4.9). *P.kawakamii* shoots formed small callus regardless of gelling agents and concentration. Gelrite induced more callus growth in *P.taiwaniana* and *P.tomentosa* than agar or agar + gelrite medium regardless of its concentration. *P.fortunei* produced more callus growth in low concentrations of agar or gelrite + agar mixture and callus growth decreased as the concentration of gelling agent increased (Table 4.10).

Vitrified shoots of *P.taiwaniana* and *P.kawakamii* produced more shoots than normal shoots (Table 4.11), but this was not the case for the other two species. This

morphological characteristic of axillary shoot induction in vitrified shoots was similar to that reported by Böttcher *et al.* (1988) for *Dianthus* shoot cultures.

Table 4.6. Anova of degree of vitrification, callus size and number of axillary shoots produced in normal and vitrified shoot explant as affected by gelling agent and species.

Source of Variance	Df	Mean squares for		
		VitNo	CallusNo	ShootNo
Replication	4	0.4	0.5	0.28
Gel[1]	9	3.5*	6.1*	0.38
Spp[2]	3	6.0*	15.6*	12.37*
N&V[3]	1	84.0*	28.2*Tab.9	32.77*
GelxSpp	27	0.8	1.0*Tab.10	0.20
GelxN&V	9	2.1*Tab.7	0.7	0.32
SppxN&V	3	3.4*Tab.8	1.6	12.29*Tab.11
GelxSppxN&V	27	0.4	0.5	0.19
Error	308	0.6	0.5	0.24

[1]Gel: gelling agents, [2]Spp: species, [2]N&V:normal & vitreous shoots, Tab.7-11: Tables 4.7-11
* represent significance at 1 % level.

Table 4.7. Degree of vitrification of normal and vitrified shoot explants cultured on different gelling agents

Shoot type	Agar (%)				Gelrite (%)			0.1 % Gelrite + Agar(%)		
	0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
Normal	0.0 e	0.0 e	0.0 e	0.0 e	0.2 de	0.2 de	0.0 e	0.0 e	0.2 de	0.0 e
Vitrified	1.5 ab	0.6 cde	0.4 de	0.3 de	1.9 a	1.5 ab	1.3 abc	0.9 bcd	1.0 bcd	0.9 bcd

Means separation by Turkey's test 1 % level

Table 4.8. Degree of vitrification from normal and vitrified shoot explants of four species of *Paulownia*

Shoot type	*P.fortunei*	*P.kawakamii*	*P.tomentosa*	*P.taiwaniana*
Normal	0.1de	0.0e	0.1de	0.0e
Vitrified	1.5a	0.9bc	1.2ab	0.5cd

Means separation by Tukey's test 1 % level

Table 4.9. Callus size per plantlet from normal and vitrified shoot explants

	Normal shoots	Vitrified shoots
Callus size	0.27b	0.83a

Means separation by Tukey's test 1 % level

Table 4.10. Callus size per plantlet as affected by gelling agent and species

Species	Agar (%)				Gelrite (%)			0.1 % Gelrite + Agar (%)		
	0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
P.fortunei	1.3 abcd	0.6 bcdef	0.2 def	0.2 def	2.2 a	1.6 ab	1.5 abc	1.5 abc	0.8 bcdef	0.6 bcdef
P.kawakamii	0.0 f	0.1 ef	0.0 f	0.0 f	0.3 def	0.3 def	0.1 ef	0.1 ef	0.1 ef	0.1 ef
P.tomentosa	0.4 bcdef	0.1 ef	0.1 ef	0.1 ef	1.8 ab	1.5 abc	1.2 bcde	1.0 bcdef	0.5 cdef	0.1 ef
P.taiwaniana	0.0 f	0.0 f	0.4 def	0.1 ef	1.1 bcde	1.1 bcde	0.6 bcdef	0.2 edf	0.5 cdef	0.0 f

Means separation by Tukey's test 1 % level

Table 4.11. Axillary shoots produced by normal and vitrified shoot explants of four species of *Paulownia*

Shoot type	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
Normal	1.0c	1.0c	1.0c	1.0c
Vitrified	1.0c	1.9b	1.0c	2.5a

Means separation by Tukey's test, 1 % level

3.3.2 Effect on the growth and water content of plantlets

Shoot dry weight produced by vitrified shoots was greater than that of normal shoots for all four species, but particularly for *P.fortunei* and *P.tomentosa* (Tables 4.12 and 13). It may be that the metabolism of vitrified shoots was higher than that of normal shoots as pointed out by Gasper *et al.* (1987). Lower concentrations of gelling agents favoured shoot growth (Table 4.14) which is in agreement with the reports of Harbaoui and Debergh (1980) and Debergh *et al.* (1981). The root dry weight produced by normal and vitrified shoots was the same (Table 4.12). *P.fortunei* produced more root dry weight in 0.6 % agar than any of the other treatments (Table 4.15). For the other three species root dry weight tended to be higher on gelrite medium with a tendency to decrease with increasing concentration of gelling agent. Plantlet dry weight of vitrified shoots was greater than that for normal shoots (Table 4.16). The increase of dry weight came from growth of the shoot rather than the root (Table 4.12). Plantlet dry weight of *P.fortunei* in 0.6 % agar medium was higher than for any of the other treatments (Table 4.17). All the species except *P.kawakamii* had a higher plantlet dry weight in lower concentrations of the gelling agent. Growth of *P.kawakamii* plantlets was affected less by gelling agent possibly due to the inherently slower growth rate of this species.

Water content (the ratio of fresh weight to dry weight of plantlets of normal shoots was not affected by gelling agent and concentration (Table 4.18). Water content of vitrified shoots decreased when agar concentration increased. This decrease in water content appeared to be related to shoots reverting to normal shoots (Table 4.18). Water content of *P.kawakamii* and *P.taiwaniana* tended to be less than that for the other two species when cultured on agar media. These differences disappeared more or less when gelrite and mixtures of gelrite and agar were used as a gelling agent (Table 4.10).

Reduction of vitrified shoots was accompanied by low rates of multiplication in this study as has frequently been reported (Gaspar *et al.*, 1987; Ziv, 1990). However, vitrified shoots of all four *Paulownia* species reverted to normal growth when subcultured on MS medium solidified with 0.8 % agar. This suggests that it is not necessary to reduce vitrified shoot formation on shoot formation medium. Irrespective of vitrified or normal adventitious shoots, healthy plantlets later can be obtained in the *in vitro* rooting stage

Table 4.12. Anova of shoot dry weight (ShtDw), root dry weight (RootDw), plant dry weight (PlantDw) and plant dry weight/plant fresh weight (PlantFDw) per normal and vitrified shoot explant as affected by gelling agents and species

Source of Variance	Df	Mean squares for			
		ShtDw	RootDw	PlantDw	PlantFDw
Replication	4	94	27	189	4.5
Gel[1]	9	1175*[T.14]	31*	1684*	7.6*
Spp[2]	3	1988*	102*	2443*	12.8*
N&V[3]	1	6938*	34	5570*[T.16]	9.2
GelxSpp	27	169	18*[T.15]	301*[T.17]	6.4*[T.18]
GelxN&V	9	156	12	231	8.0*[T.18]
SppxN&V	3	506*[T.13]	17	357	4.4
GelxSppxN&V	27	92	12	88	1.4
Error	308	122	10	151	2.3

[1]Gel: gelling agents, [2]Spp: species, [3]N&V:normal & vitreous shoots, T.13-18: Tables 4.13-18

* represent significance at 1 % level.

Table 4.13. Shoot dry weight (mg) per plantlet for normal and vitrified shoot explants for 4 species of *Paulownia*

Shoot type	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
Normal	19ef	17f	24cd	21def
Vitrified	29b	22cde	37a	26bc

Means separation by Tukey's test, 1 % level

Table 4.14. Shoot dry weight (mg) per plantlet as affected by gelling agent and concentration

Agar (%)				Gelrite (%)			0.1 % Gelrite + agar (%)		
0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
32a	20bcd	18cd	16d	32a	27ab	23bcd	28ab	26abc	20bcd

Means separation by Tukey's test, 1 % level

Table 4.15. Root dry weight (mg) per plantlet as affected by gelling agent and concentration for 4 species of *Paulownia*

Species	Agar (%)				Gelrite (%)			0.1%Gelrite + Agar(%)		
	0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
fortunei	12.9 a	2.6 cd	1.5 d	1.6 d	1.0 d	1.2 d	1.5 d	1.9 d	2.2 d	1.0 d
kawakamii	2.2 d	1.2 d	1.7 d	1.5 d	3.5 bcd	3.5 bcd	2.0 d	2.0 d	1.8 d	2.6 cd
tomentosa	4.2 bcd	3.0 cd	1.9 d	1.8 d	7.7 bc	3.8 bcd	3.6 bcd	2.2 d	4.6 bcd	2.8 cd
taiwaniana	3.5 bcd	2.4 d	1.9 d	2.4 d	6.2 bc	3.0 cd	3.9 bcd	4.4 bcd	8.2 b	5.8 bcd

Means separation by Tukey's test, 1 % level

Table 4.16. Plantlet dry weight (mg) of normal and vitrified shoot explants per plantlet between normal and vitrified shoots

	Normal shoots	Vitrified shoots
Plantlet dry weight (mg)	24b	31a

Means separation by Tukey's test, 1 % level

Table 4.17. Plantlet dry weight (mg) per plantlet as affected by gelling agent and concentration for 4 species of *Paulownia*

Species	Agar (%)				Gelrite (%)			0.1%Gelrite + Agar(%)		
	0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
P.fortunei	55	25	16	16	37	26	24	30	24	15
P.kawakamii	22	17	20	17	25	24	18	25	25	24
P.tomentosa	40	34	22	23	47	37	34	39	37	27
P.taiwaniana	32	18	21	17	35	32	27	30	36	27

Least significant differences of Tukey T test 1 % level: 15

Table 4.18. Water content (Fw/Dw) per plantlet produced by normal and vitrified shoot explants and as affected by gelling agent and concentration in 4 species of *Paulownia*

Water content	Agar (%)				Gelrite (%)			0.1 % Gelrite + Agar(%)		
	0.6	0.8	1.0	1.2	0.2	0.3	0.4	0.3	0.4	0.5
Shoot type										
Normal	10.2 abcd	10.4 abcd	9.9 bcd	9.9 bcd	10.0 bcd	9.9 bcd	10.7 abc	10.0 bcd	10.1 bcd	9.8 bcd
Vitrified	11.0 abc	9.1 d	9.7 bcd	9.4 cd	11.7 a	11.2 abc	10.7 abc	10.9 abc	10.3 abcd	9.8 bcd
Species-										
fortunei	11.7 abc	11.0 abcd	10.5 bcd	9.7 cd	9.9 cd	11.0 abcd	10.4 bcd	12.5 ab	10.8 abcd	9.5 cd
kawakamii	10.1 cd	9.6 d	9.2 d	9.6 cd	10.6 bcd	10.4 bcd	10.6 bcd	10.4 bcd	10.2 cd	9.4 cd
tomentosa	11.3 abc	9.3 d	10.1 cd	10.2 cd	12.8 a	10.2 cd	10.9 abcd	9.6 cd	9.4 cd	10.0 cd
taiwaniana	9.2 d	9.3 d	9.5 cd	9.0 d	10.1 cd	10.5 bcd	11.0 abcd	9.3 d	10.5 bcd	10.2 cd

Means separation by Tukey's test, 1 % level

4. REFERENCES

Bornman, C.H. and Bogelmann, T.C., 1984. Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification in *in vitro Picea abies*. Physiol. Plant. 61:505-512

Böttcher, I., Zoglauer, K. and Göring, H., 1988. Induction and reversion of vitrification of plants cultured *in vitro*. Physiol. Plant. 72:560-561

Debergh, P.C., 1983. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59:270-276

Debergh, P.C., 1987. Improving micropropagation. IAPTC Newsletter 51:2-10

Debergh, P.C., Harbaoui, Y. and Lemeur, R., 1981. Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53:181-187

Gaspar, T. and Kevers, C., 1985. Cobalt prevention of vitrification process in carnation. Plant Physiol. 77 (supl.):13

Gaspar, T., Kevers, C., Debergh, P., Maene, L., Paques, M. and Boxus, P., 1987. Vitrification: morphological, physiological, and ecological aspects. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry. Vol.1: General principles and biotechnology. (pp 152-166) Martinus Nijhoff Publishers, The Netherlands

George, E.F. and Sherrington P.D., 1984. Plant propagation by tissue culture: Handbook and directory of commercial laboratories. (709 pp.) Eastern Press, England

Goldfarb, , Howe, G.T., Bailey, L.M., Strauss, H. and Zaerr, J.B., 1991. Liquid cytokinin pulse induces adventitious shoot formation from Douglas-fir cotyledons. Plant Cell Rep. 10:156-160

Harbaoui, Y. and Debergh, P., 1980 Dore, C. (Ed.) Application de la culture *in vitro* á l'amélioration des plantes potagères (pp. 1-7) Eucarpia meeting, Versailles. Multiplication *in vitro* de clones sélectionnés d'artichaut (*Cynara scolymus* L.). In: Debergh, P.C., Harbaoui, Y. and Lemeur, R., 1981. Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53:181-187

Ho, C.K., Chang, S.H. and Yang, J.C., 1988. Tissue culture of *Paulownia taiwaniana* from juvenile tissues. Symposium of Tree Improvement and Tissue Culture in Taiwan. (pp. 97-113) Experimental Forest of National Taiwan University

Leshem, B., 1983. The carnation succulent plantlet - a stable teratological growth. Ann. Bot. 52:873-876

MacRae, S. and Van Staden, J., 1980. *In vitro* culture of *Eucalptus grandis*: Effect of gelling agents on propagation. J. Plant Physiol. 137:249-251

McCown, D.D. and McCown, B.B., 1987. North American hardwood. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry Vol.3: Case

histories: gymnosperms, angiosperms and plams (pp.253) Martinus Nijhoff Publishers, The Netherlands

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479

Nairn, B.J., 1987 Significance of gelling agents in a production tissue culture laboratory. Combined Proc. Int. Plant Prop. Soc. 37:200-205

Orlikowska, T., 1987. Vitrification problem in the *in vitro* culture of fruit tree rootstocks. Acta Hort. 212:239-244

Pasqualetto, P.L., Zimmerman, R.H. and Fordham, I., 1986. The influence of gelling agent and growth regulator concentrations on vitrification of apple cultivars *in vitro*. J. Amer. Soc. Hort. Sci. 111:976-980

Pasqualetto, P.L., Zimmerman, R.H. and Fordham, I., 1988 The influence of cation and gelling agent concentration on vitrification of apple cultivars *in vitro*. Plant Cell, Tissue & Organ Cult. 14:31-40

Phan, C.T., 1991. Vitreous state *in vitro* culture: ethylene versus cytokinin. Plant Cell Rep. 9:517-519

Rugini, E., Tarini, P. and Rossodivita, M.E., 1987. Control of shoot "vitrification" of almond and olive grown *in vitro*. Acta Hort. 212:177-183

Van Arnold, S. and Eriksson, T., 1984. Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. Plant Cell, Tissue & Organ Cult. 3:257-264

Williams, R.A. and Taji, A.M. 1991. Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* (J.M. Black) *in vitro* shoot cultures. Plant Cell, Tissue & Organ Cult. 26:1-6

Zimmerman, T.W. and Cobb, B.G., 1989. Vitrification and soluble carbohydrate levels in *Petunia* leaves as influenced by media gelrite and sucrose concentrations. Plant Cell Rep. 8:358-360.

Ziv, M., 1990. Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh, P.C. and Zimmerman, R.H. (Eds.) Micropropagation: Technology and application (pp 45-69) Kluwer Academic Publishers, The Netherlands

Ziv, M., Meir, G. and Halevy, A.H., 1981. Hardening carnation plantlets regenerated from shoot tips cultured *in vitro*. Environ. Exp. Bot. 21:423

Ziv, M., Meir, G. and Halevy, A.H., 1983. Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. Plant Cell, Tissue & Organ Cult. 2:55-60

CHAPTER 5

INDUCTION OF SOMATIC EMBRYOGENESIS

key words: embryo culture, ovule culture, seed culture, somatic embryogenesis

ABSTRACT

Induction of somatic embryogenesis in four species of *Paulownia* was investigated. Ovules of *P.fortunei* at the globular proembryo stage cultured on Radojevic (1979) , MSG (Amerson *et al.*, 1988) and DCR (Gupta & Durzan, 1986a) media supplemented with auxins and cytokinins developed into mature embryos or formed callus. Neither callus nor embryos developed further when subcultured. Mature and immature seeds cultured on various media germinated in low numbers. Germinating embryos were cultured on Radojevic medium or MGM medium (Muralidharan *et al.*, 1989) supplemented with 2, 5 % sucrose and picloram, 2,4-D or NAA combined with kinetin or BA in light or dark failed to form embryogenic tissue. Embryogenesis could not be induced in *P.tomentosa* by the procedure described by Radojevic (1979).

ABBREVIATIONS USED

BA = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid, NAA = α-naphthaleneacetic acid, picloram = 4-amino-3,5,6-trichloropicolinic acid

1. INTRODUCTION

Somatic embryogenesis has been induced in more than 25 families, 44 genera, 60 species and numerous cultivars of trees. In 33 species somatic embryogenesis have developed into plantlets which have been planted out in the ground (Tulecke, 1987). Since then plants have been regenerated from somatic embryogenesis in many more species of woody plants such as *Picea abies* (Gupta & Durzan, 1986b; Becwar *et. al.*, 1987a), *Larix* x *eurolepis* (Klimaszewska, 1989), *Picea glauca* (Lu & Thorpe, 1987; Hakman & Fowke, 1987; Tremblay, 1990), *Picea mariana* (Hakman & Fowke, 1987), *Pinus taeda* (Gupta & Durzan, 1987), *Pinus strobus* (Finer *et al.*, 1989), *Picea engelmarii* (Webster *et al.*, 1990), *Quercus robur* and *Tilia cordata* (Chalupa, 1990), *Populus ciliata* (Cheema, 1989), *Rosa* 'Domingo' and 'Vickey Brown' (De Wit *et al.*, 1990), *Rhododendron laetum* x *aurigeranum* (Iapichino *et al.*, 1991), *Cocos nucifera* (Karunaratne & Periyapperuma, 1989), *Eucalyptus citriodora* (Muralidharan *et al.*, 1989), *Olea europea* var. *sylvestris* (Orinos & Mitrakos, 1991), *Rauvolfia vomitoria* (Trémouillauz-Guiller & Chénieux, 1991), *Camellia japonica* (Vieitez & Barciela, 1990), *Carica papaya* (Fitch & Manshardt, 1990). Somatic embryogenesis of forestry trees can be useful for large scale propagation and to serve as a source of totipotent material for protoplast culture and direct gene transfer (Debergh, 1989). Tree breeding will advance greatly if foreign DNA can be introduced into already proven genotypes without loosing the parental traits (McCown, *et al.*, 1991). Somatic embryogenesis of *P.tomentosa* has been induced from ovules and germinating zygotic embryos (Radojevic, 1979). Embryogenic callus of fertilized ovules has retained their ability to form somatic embryos for four years, however, they failed to grow into plants. Embryogenic callus from germinating seedlings developed into plantlets that could not be maintained for more than three months (Radojevic, 1979). In this study the experiments of Radojevic on induction of somatic embryogenesis of *P.tomentosa* were repeated and extended to 3 other species of *Paulownia*.

2. MATERIAL AND METHODS

2.1 Ovule culture

Ovaries (ca 7 mm in length) with fertilized ovules were collected from a 8 year-old *P.fortunei* tree growing at the Forestry Faculty of University of Stellenbosch. Ovaries were sterilized with alcohol (70 % for 1 min) and commercial bleach (3.5 % NaOCl, plus one drop of Tween 20 per 100 ml for 15 min) and then rinsed three times with sterile water. Placentas bearing ovules in the globular proembryo stage were excised from ovaries and cultured. Two placentas were used per 100 ml flask containing 20 ml liquid media while one placenta was used per test tube containing 5 ml media solidified with 0.7 % Difco agar. Liquid cultures were placed on a reciprocal shaker at 100 rpm and kept in a dark room. Solid cultures in test tubes were kept in a culture room at 25^{o}C with 16 hour photoperiod of 30 $\mu Em^{-2}s^{-1}$ light intensity or in a dark room at 25^{o}C. Culture media used were: (1) Radojevic's (1979) (R) basal medium which consisted of MS medium (Murashige & Skoog, 1962) plus 2 % sucrose, 200 mg l^{-1} casein hydrolysate, 100 mg l^{-1} myoinositol, 2 mg l^{-1} adenine, 10 mg l^{-1} pantothenic acid (R medium) and supplemented with combinations of IAA (0.1, 1.0, 3.0 mg l^{-1}) and kinetin (0.1, 1.0 mg l^{-1}), (2) R medium containing combinations of 2,4-D (0.1, 1.0 mg l^{-1}) and kinetin (0.1, 1.0 mg l^{-1}). (3) MSG basal medium (Amerson *et al*, 1988) supplemented with 3 g l^{-1} sucrose, 1.45 g l^{-1} glutamine, 0.7 % Difco agar, and combinations of 2,4-D (1, 5, 10 mg l^{-1}) and BA (0.1, 1 mg l^{-1}), (4) DCR basal medium (Gupta & Durzan, 1986a) supplemented with 3 g l^{-1} sucrose, 0.25 g l^{-1} glutamine, 0.7 % Difco agar, and combinations of 2,4-D (2, 10, 20 mg l^{-1}) and BA (0.2, 2 mg l^{-1}). Treatments were repeated three times. Ovule development was studied every week under a stereo microscope. After 5 weeks, callusing embryos or developing embryos were subcultured on to the same medium.

2.2 Seeds and embryo culture

Mature seeds of *P.fortunei*, *P.tomentosa*, *P.kawakamii* and *P.taiwaniana* and green immature capsules of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* were sterilized as described in section 2.1. Unlike mature seeds, immature seeds which were attached to the placenta had a high water content and the colour was lighter than that of mature seeds. Sterilized mature seeds (ca 50) were placed in 220 ml jars containing 30 ml water solidified with 0.6 % agar. As soon as the embryos ruptured the seed coats they were transferred into test tubes containing 5 ml of culture media. Two germinating embryos were cultured per test tube. Immature seeds (ca 10) were also placed into test tubes containing 5 ml of the same media. Media used were: (1) R medium supplemented with combinations of IAA (0.1, 0.5, 1.0 mg l^{-1}) and kinetin (0.5, 1.0, 2.5, 5.0 mg l^{-1}) and 0.7 % agar; (2) R medium supplemented with combinations of picloram (0.1, 0.5, 1.0 mg l^{-1}) and BA (0.5, 1.0, 2.5, 5.0 mg l^{-1}) and 0.7 % agar; (3) R medium supplemented with 2,4-D (0.1, 0.5, 1.0 mg l^{-1}) and BA (0.5, 1.0, 2.5, 5.0 mg l^{-1}) and 0.7 % agar; (4) Muralidharan *et al.* (1989) basal medium which consisted of B5 medium of Gamborg *et al.* (1968) plus 5 % sucrose, 500 mg l^{-1} glutamine, 50 mg l^{-1} casein hydrolysate, coconut milk 10 % (MGM medium). The MGM medium was supplemented with 0.2 % gelrite and combinations of 2,4-D (1, 2, 4 mg l^{-1}) and BA (0, 0.1, 1 mg l^{-1}); (5) MGM medium containing combinations of NAA (1, 2, 4 mg l^{-1}) and BA (0, 0.1, 1.0 mg l^{-1}) and 0.2 % gelrite. Cultures were kept in light or dark as described in ovule cultures. Treatments were repeated five times in R medium and four times in MGM medium. Organogenesis and callus growth were followed over a 5 week period. Callus sizes were rated as -, +, ++, +++ for none, small, medium or large.

3. RESULTS AND DISCUSSION

3.1 ovule culture

In liquid culture of R medium containing 3 mg l^{-1} IAA and 0.1 mg l^{-1} kinetin or 1 mg l^{-1} 2,4 D and 0.1 mg l^{-1} kinetin, MSG medium containing 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA, and DCR medium containing 2 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA, ovaries gradually released from the placentas (Fig. 5.1-a). Different developmental patterns of the ovules were observed. Some developed into torpedo-shaped embryos with an apical and radical meristem (Fig. 5.1-b). Others formed seed coats and wings without embryos (Fig 5.1-c). A third group developed into apparently normal seeds with testae and wings (Fig 5.1-d), while compact globular callus was formed by some of the ovules. Subculture of these structures failed to develop further, they turned brown and died. On solid medium in the dark only a few ovules grew and formed callus. In light , ovules developed in different ways. In some cases a small number of ovules on a placenta grew and formed friable and water-soaked callus (Fig 5.2-a); in other cases many ovules on a placenta formed callus (Fig 5.2-b); occasionally, embryos with two cotyledons formed (Fig 5.2-c). Growth of ovaries occurred predominantly in medium containing 1 or 2 mg l^{-1} 2,4-D or 3 mg l^{-1} IAA and 0.1 mg l^{-1} kinetin or 0.1, 0.2 mg l^{-1} BA irrespective of the different basal media. Subcultured ovaries failed to develop further as was the case in liquid culture. Considerable variation in developmental patterns of ovaries was observed in the same medium. This is probably due to different developmental stages of the ovules within an ovary as has been reported for other species (James *et al.*, 1984; Lu & Thorpe, 1987; Hakman & Fowke, 1987; Becwar *et al.*, 1987b; Feirer *et al.*, 1989; Zimny & Lorz, 1989; Chalupa, 1990; Sotak *et al.*, 1991). In *Paulownia* pollen tube growth takes 5-7 days and fertilization takes place 7-10 days after pollination (Millsaps, 1936; Chinese Academy of Forestry & Honan

Forestry Bureau, 1978; Chinese Academy of Forestry 1986). The flowering period lasts 1 month and occurs in April (northern hemisphere) or September (southern hemisphere). After fertilization, the endosperm nucleus divides and the embryo sac is filled with endosperm. At this stage the embryo is globular in shape. Embryo development continues and seeds mature in August (northern hemisphere) or April/May (southern hemisphere). Radojevic (1979) reported embryogenesis in ovule culture of *P.tomentosa*, but did not specify the embryo stage used.

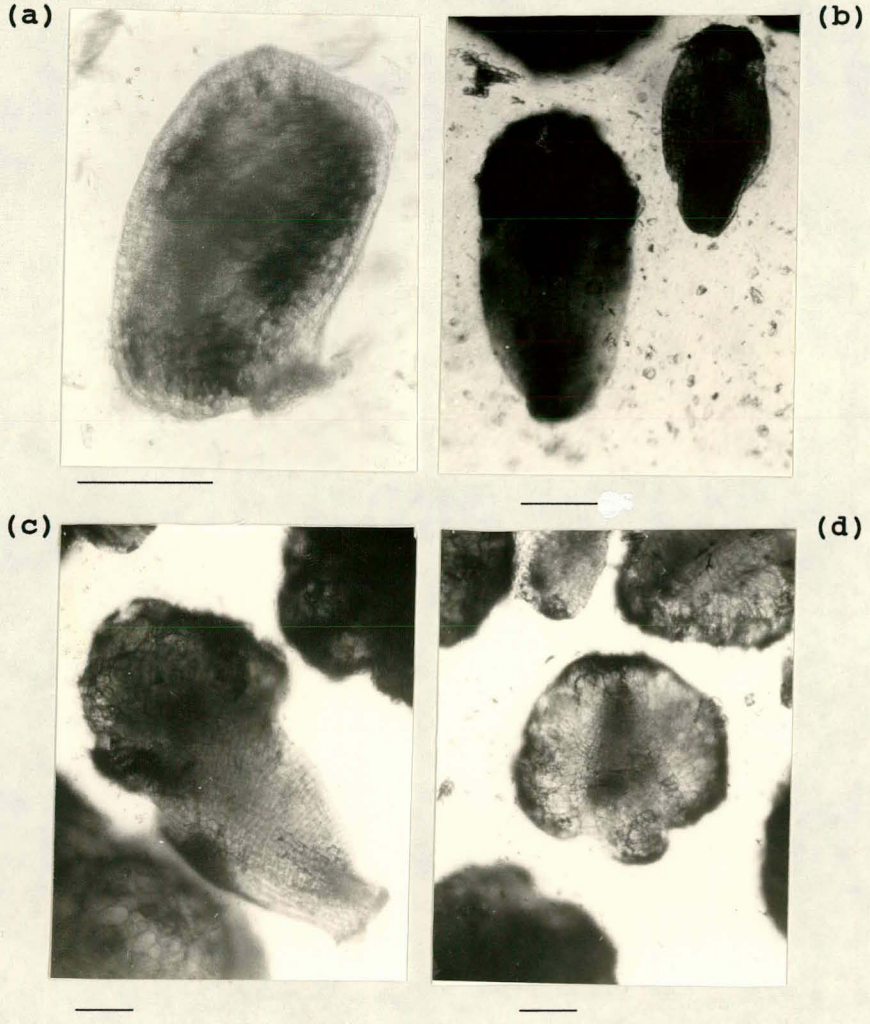


Fig 5. 1. Placenta cultures of *P.fortunei* in liquid R medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin: (a) ovule released from placenta, (b) torpedo shaped embryo with dense apical and radicle meristems, (c) testa and seed wings without embryo, (d) seed with two wings comparable in shape but smaller than mature seed. Bars represent 0.5 mm.

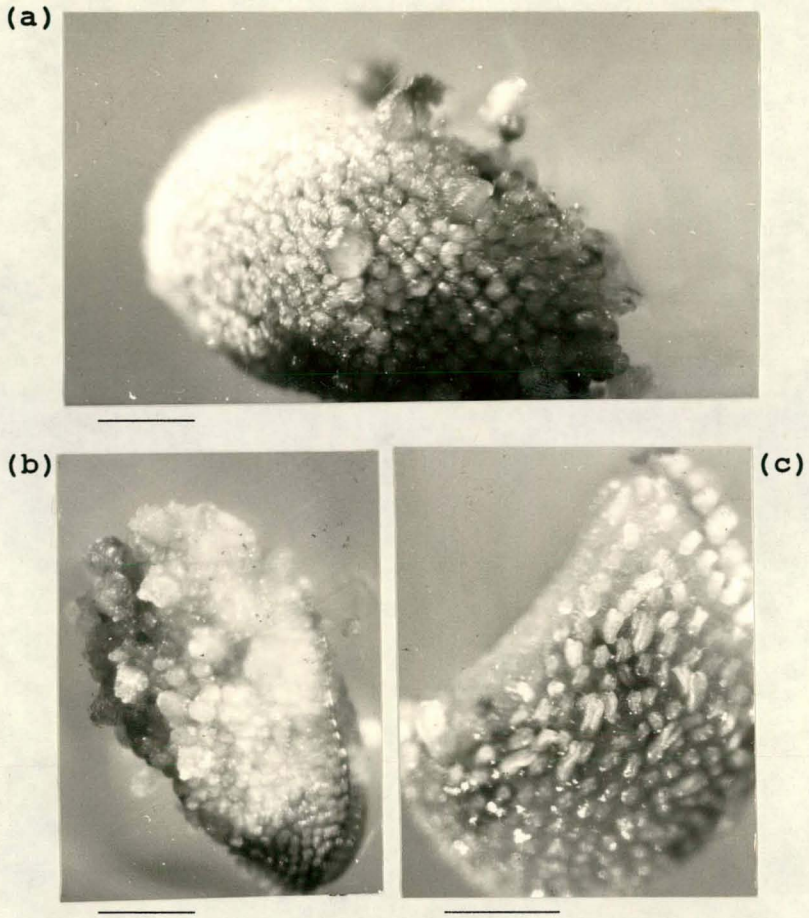


Fig 5. 2. Developmental patterns of placentas cultured on solid R or MSG or DCR medium supplemented with 1, 2 mg l^{-1} 2,4-D or 3 mg l^{-1} IAA and 0.1 mg l^{-1} kinetin or 0.1, 0.2 mg l^{-1} BA: (a) few ovules with callus, (b) many ovules formed callus, (c) torpedo shaped embryos. Bars represent 1 mm.

3.2 Seed and embryo culture

Poor results were obtained with mature and immature seeds cultured in the different media both in light and in dark. Poor germination of seeds is probably related to the inhibitory effect of MS medium as reported in Chapter 2. However, some immature seeds in the dark germinated and formed small grey callus in MGM medium containing 2,4-D and BA or R medium containing 2,4-D and kinetin (Table 5.1). Normal germination and seedling growth occurred when immature seeds were cultured in R medium containing IAA and kinetin in light. Embryos are commonly used to induce embryogenesis in woody plants (Tulecke, 1987). However, no somatic embryogenesis from germinating embryos of mature seeds was induced in these experiments despite the wide range and combination of different auxins and cytokinins used (Tables 5.2 - 5). Picloram is comparable or superior to NAA or 2,4-D for long term shoot multiplication and induction of somatic embryogenesis (Phillips & Luteyn, 1983). Combinations of 2,4-D and BA are most commonly used in woody plants to induce somatic embryogenesis (Tulecke, 1987). Picloram or 2,4-D combined with BA in light induced friable callus growth in three species of *Paulownia* (Tables 5.2 and 5.3). Embryogenic callus formed from embryos of *P.tomentosa* cultured on IAA and kinetin (Radojevic, 1979). In this study combinations of IAA and kinetin induced adventitious buds and limited callus growth. Most embryos of *P.kawakamii* and *P.tomentosa* grew normally into plants (Table 5.4). Adventitious buds formed on hypocotyls and cotyledons of *P.tomentosa* in embryos cultured on high IAA levels whereas for *P.kawakamii* adventitious buds formed in high kinetin levels. The MGM medium containing NAA and BA induced somatic embryogenesis in *Eucalyptus citriodora* (Muralidharan *et al.*, 1989) but in *Paulownia* callus growth and roots were induced (Table 5.5). High sucrose concentrations (from 4 to 12 %) improved somatic embryogenesis in many species (Ammirato, 1983; Close & Ludeman, 1987; Lu &

Thorpe, 1987; Jain *et al.*, 1988; Brown *et al.*, 1989; Finer *et al.*, 1989; Muralidharan *et al.*, 1989; Gavish *et al.*, 1991; Tremblay & Tremblay, 1991). In these experiments sucrose concentrations varied from 2 % in R medium to 5 % in MGM medium. Higher concentrations of sucrose should therefore also be tested for *Paulownia*.

Table 5.1. Effect of 2,4-D and BA or kinetin in MGM medium on germination and callus growth and colour of immature seeds of *Paulownia* cultured in dark

2,4-D mg l^{-1}	Cytokinins mg l^{-1}	*P.fortunei*		*P.kawakamii*		*P.taiwaniana*	
		Germ%	Callus	Germ%	Callus	Germ%	Callus
1	0	4	+ brown	0	-	0	-
2	0	2	+ brown	0	-	0	-
4	0	2	+ brown	0	-	6	+ brown
------	**BA**	----------		----------		----------	
1	0.1	6	+ brown	2	+ brown	0	-
2	0.1	6	brown	0	-	2	+ brown
4	0.1	0	-	0	-	14	+ brown
1	1.0	2	++ grey	4	+ brown	4	+ brown
2	1.0	2	+ brown	0	-	8	- brown
4	1.0	14	+ brown	0	-	0	-
----	**kinetin**	----------		----------		----------	
1	0.1	4	+ brown	4	+ brown	10	+ grey
2	0.1	4	++ grey	0	-	16	++ grey
4	0.1	0	-	0		2	- brown
1	1.0	20	+++ grey	0	-	0	-
2	1.0	8	+++ grey	0	-	0	-
4	1.0	2	+ grey	0	-	0	-

-, +, ++, +++ represent callus size as none, small, medium or large

Table 5.2. Effect of Picloram and BA in R medium on the callus growth from embryos of *Paulownia* cultured in light

Picloram mg l^{-1}	BA mg l^{-1}	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
0.1	0.5	+++	++	++++	-
	1.0	+++	++	++++	+
	2.5	+++	+++	+++	++
	5.0	+++	+++	++++	-
0.5	0.5	++	+	++	-
	1.0	+	+	++	-
	2.5	+++	++	++	+
	5.0	+++	++	++	-
1.0	0.5	+	+	++	-
	1.0	+	+	++	+
	2.5	+	+	++	+
	5.0	+	+	++	-

-, +, ++, +++ represent callus size as none, small, medium or large

Table 5.3. Effect of 2,4-D and BA in R medium on callus growth of embryos of *Paulownia* in light

2,4-D mg l^{-1}	BA mg l^{-1}	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
0.1	0.5	++	+++	+++	+
	1.0	+	+++	+++	+
	2.5	-	+++	+++	+
	5.0	+++	++	+++	-
0.5	0.5	++	++	+++	-
	1.0	+++	++	++	-
	2.5	++	++	+++	-
	5.0	++	++	++	+
1.0	0.5	+	+	+	-
	1.0	+	+	+	+
	2.5	+	+	+	-
	5.0	+	+	+	-

-, +, ++, +++ represent callus size as none, small, medium or large

Table 5.4. Effect of IAA and kinetin in R medium on plant growth and adventitious bud formation of embryos of *P.kawakamii* and *P.tomentosa* cultured in light

IAA mg l^{-1}	kinetin mg l^{-1}	*kawakamii*		*tomentosa*	
		Sht ht (mm)	AB/seedling	Sht ht (mm)	AB/seedling
0.1	0.5	16.4	0	25.4	0
	1.0	17.4	0	28.8	0
	2.5	17.4	0	37.4	1.2 (hyp)
	5.0	22.0	1.0 (cot)	45.8	0.2 (hyp)
0.5	0.5	18.6	0	34	0
	1.0	12.6	0	30.6	0
	2.5	19.4	0	41.4	0.6 (cot)
	5.0	15.4	0.8 (cot)	46.2	2.8 (hyp)
1.0	0.5	28.0	0	31.2	0.6 (hyp)
	1.0	24.0	0	34.4	1.8 (hyp)
	2.5	16.2	0	31.8	0.6 (hyp)
	5.0	13.0	1.2 (hyp)	41.0	2.8 (hyp)

Sht ht: shoot height, AB: adventitious bud, cot & hyp: adventitious buds induced from cotyledon or hypocotyl

Table 5.5. Effect of NAA and BA in MGM medium on the callus growth of embryos of *Paulownia* cultured in dark

NAA mg l^{-1}	BA mg l^{-1}	*fortunei*	*kawakamii*	*tomentosa*	*taiwaniana*
1	0	-	++	++	+
2	0	-	++	++	++
4	0	-	+	++	++
1	0.1	-	++	++	+
2	0.1	+++	+++	+++	+++
4	0.1	+	++	+++	+++
1	1.0	-	+	++	+
2	1.0	-	+	++	++
4	1.0	-	++	++	+

-, +, ++, +++ represent callus size as none, small, medium or large

4. REFERENCES

Amerson, H.V., Frampton, L.J., Mott, R.L. and Spaine, P.C., 1988. Tissue culture of conifers using loblolly pine as a model. In: Hanover, J.W. and Keathley, D.E., (Eds.) Genetic manipulation of woody plants. (pp. 117-137) Plenum Press, New York

Ammirato, P.V., 1983. Embryogenesis. In: Evans, D.A., Sharp, W,R,, Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture Vol.1: Techniques for propagation and breeding. (pp. 82-123) Macmillan Publishers Co., New York

Becwar, M.R., Nolant, T.L. and Wann, S.R., 1987a. Somatic embryo development and plant regeneration from embryogenic Norway spruce callus. Tappi J. 70:155-160

Becwar, M.R., Noland, T.L., Wann, S.R., 1987b. A method for quantification of the level of somatic embryogenesis among Norway spruce callus lines. Plant Cell Rep. 6:35-38

Brown, C., Brooks, F.J., Pearson, D. and Mathias, R.J., 1989. Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic acid. J. Plant Physiol. 133:727-733

Chalupa, V., 1990. Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.) Plant Cell Rep. 9:398-401

Cheema, G.S., 1989. Somatic embryogenesis and plant regeneration from cell suspension and tissue cultures of mature himalayan poplar (*Populus ciliata*). Plant Cell Rep. 8:124-127

Chinese Academy of Forestry, 1986. *Paulownia* in China: Cultivation and utilization. (65 pp.) Asian Network for Bio. Sci. & Int. Develop. Res. Centre

Chinese Academy of Forestry and Honan Forestry Bureau, 1978. Research on *Paulownia*. (266 pp.) Chinese Forestry Publishers.

Close, K.R. and Ludeman, L.A., 1987. The effect of auxin-like plant growth regulators and osmotic regulation on induction of somatic embryogenesis from elite maize inbreds. Plant Sci. 52:81-89

De Wit, J.C., Esendam, H.F., Honkanen, J.J. and Tuominen, U., 1990. Somatic embryogenesis and regeneration of flowering plants in rose. Plant Cell Rep. 9:456-458

Debergh, P., 1989. Recent progress in somatic embryogenesis. Newsl., Int. Assoc. for Plant Tissue Cult. 57:3-16

Feirer, R.P., Conkey, J.H. and Verhagen, S.A., 1989. Triglycerides in embryogenic conifer calli: A comparison with zygotic embryos. Plant Cell Rep. 8:207-209

Finer, J.J., Kreibel, H.B. and Becwar, M.R., 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Rep. 8:203-206

Fitch, M.M.M. and Manshardt, R.M., 1990. Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). Plant Cell Rep. 9:320-324

Gamborg, O.L., Miller, R.A. and Ljima, K., 1968. Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158

Gavish, H., Vardi, A. and Fluhr, R., 1991. Extracellular proteins and early embryo development in *Citrus* nucellar cell cultures. Physiol. Plant. 82:606-616

Gupta, P.K. and Durzan, D.J., 1986a. Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziessii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4:177-179

Gupta, P.K. and Durzan, D.J., 1986b. Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). In Vitro 22:685-688

Gupta, P.K. and Durzan, D.J., 1987. Somatic embryos from protoplasts of loblolly pine proembryonal cells. Bio/Tech. 5:710-712

Hakman, I. and Fowke, L.C., 1987. Somatic embryogenesis in *Picea glauca* (white spruce) and *P.mariana* (black spruce). Can. J. Bot. 65:656-659

Iapichino, G., Chen, T.H.H. and Fuchigami, L.H., 1991. Plant regeneration from somatic tissue of *Rhododendron laetum* x *aurigeranum*. Plant Cell, Tissue & Organ Cult. 27:37-43

Jain, S.M., Newton, R.J. and Soltes, E.J., 1988. Enhancement of somatic embryogenesis in Norway spruce (*Picea abies* L.). Theor. Appl. Genet. 71:501-506

James, D.J., Passey, A.J. and Deeming, D.C., 1984. Adventitious embryogenesis and the *in vitro* culture of apple seed parts. J. Plant Physiol. 115:217-229

Karunaratne, S. and Periyapperuma, K.A., 1989. Culture of immature embryos of coconut, *Cocos nucifera* L: callus proliferation and somatic embryogenesis. Plant Sci. 62:247-253

Klimaszewska, K., 1989. Recovery of somatic embryos and plantlets from protoplast cultures of *Larix* x *eurolepis*. Plant Cell Rep. 8:440-444

Lu, C-Y. and Thorpe, T.A., 1987. Somatic embryogenesis and plantlet regeneration in cultured immature embryos of *Picea glauca*. J. Plant Physiol. 128:297-302

McCown, B.H., McCabe, D.E., Russell, D.R., Robison, D.J., Barton, K.A. and Raffa, K.F., 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep. 9:590-594

Millsaps, V., 1936. The structure and development of the seed of *Paulownia tomentosa*. J. Elisha Mitchell Sci. Soc. 52:56-76

Muralidharan, E.M., Gupta, P.K. and Mascarenhas, A.E., 1989 Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. Plant Cell Rep. 8:41-43

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-479

Orinos, T. and Mitrakos, K., 1991. Rhizogenesis and somatic embryogenesis in calli from wild olive (*Olea europea* var. *sylvestris* (Miller) Lehr) mature zygotic embryos. Plant Cell, Tissue & Organ Cult. 27:183-187

Phillips, G.C. and Luteyn, K.J., 1983. Effect of picloram and other auxins on onion tissue cultures. J. Am. Soc. Hort. Sci. 108:948

Radojevic, L., 1979. Somatic embryos and plantlets from callus cultures of *Paulownia tomentosa* Steud. Z. Pflanzenphysiol. 91:57-62

Sotak, R.J., Sommer, H.E. and Merkle, S.A., 1991. Relation of the developmental stage of zygotic embryos of yellow-poplar to their somatic embryogenic potential. Plant Cell Rep. 10:175-178

Tremblay, F.M., 1990. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68:236-242

Tremblay, L. and Tremblay, F.M., 1991. Carbohydrate requirements for the development of black spruce (*Picea mariana* (Mill.) B.S.P.) and red spruce (*P.rubens* Sarg.) somatic embryos. Plant Cell, Tissue & Organ Cult. 27:93-103

Trémouillauz-Guiller, J. and Chénieux, J-C., 1991. Somatic embryogenesis from leaf protoplasts of *Rauvolfia vomitoria* shoot cultures. Plant Cell Rep. 10:102-105

Tulecke, W., 1987. Somatic embryogenesis in woody perennials. In: Bonga, J.M. and Durzan, D.J. (Eds.) Cell and tissue culture in forestry Vol.2: Specific principle and methods: growth and developments. (pp. 61-91) Martinus Nijhoff Publishers, The Netherlands

Vieitez, A.M. and Barciela, J., 1990. Somatic embryogenesis and plant regeneration from embryogenic tissues of *Camellia japonica* L. Plant Cell, Tissue & Organ Cult. 21:267-274

Webster, F.B., Roberts. D.R., Mclnnis, S.M. and Sutton, B.C.S., 1990. Propagation of interior spruce by somatic embryogenesis. Can. J. For. Res. 20:1759-1765

Zimny, J. and Lorz, H., 1989. High frequency of somatic embryogenesis and plant regeneration of rye (*Secale cereale* L.). Plant breeding 102:89-100

CHAPTER 6

REGENERATION OF CALLUS CULTURES

Key words: antioxidants, brown callus, callus regeneration, subculture

ABSTRACT

The behavior of callus tissue derived from different explant types of four *Paulownia* species cultured on MS medium plus various combinations of NAA + BA or 2,4-D + kinetin was studied in MS media containing NAA or 2,4-D plus BA or kinetin. Organogenetic callus compact in structure and greenish white in colour were induced in low levels of NAA (< 0.5 mg l^{-1}) or in combinations of NAA and BA or in low levels of 2,4-D plus kinetin. Friable callus, white or grey in colour developed in high levels of 2,4-D (0.1 mg l^{-1}) plus kinetin. Subcultures of organogenetic calli derived from a medium (0.5 mg l^{-1} NAA and 5 mg l^{-1} BA) which induced adventitious bud formation could not be maintained when subcultured. Organogenetic callus derived from a medium containing 0.1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin which caused few adventitious buds to form were induced to form many adventitious buds when subcultured on 0.01 mg l^{-1} NAA and 10 mg l^{-1} BA in the case of *P.taiwaniana* but not for *P.kawakamii*. Most callus whether organogenetic or friable turned brown when subcultured.. Treatments with antioxidants did not reduce browning. Browning of cultures with adventitious buds was less evident than in cultures without adventitious buds.

ABBREVIATIONS USED

BA = Benzylaminopurine, 2,4-D = 2,4-dichlorophenoxyacetic acid, DTT = Dithiothretiol, Kin = kinetin (6-furfuryl- aminopurine), NAA = Naphthaleneacetic acid, Polyclar = Polyvinylpyrrolidone (insoluble), PVP = polyvinylpyrrolidone (soluble)

1. INTRODUCTION

Callus cultures are generally classified as either developmental or proliferative; i.e. the callus is developing through organogenesis or embryogenesis, or it is growing and expanding in cell size and number (Keese *et al.*, 1991). Callus cells and regenerated plants from callus are known to be genetically variable or unstable (Ogura, 1990). Selection of cells tolerant or resistant to phytotoxins, herbicides, nematodes, viruses, and salts is a powerful method in plant breeding (Bajaj, 1990). In forest trees such as *Paulownia tomentosa*, *Pinus taede* and *P.caribaea* it has been shown that callus-derived plants that differ in chromosome number and DNA content (Renfroe & Berlyn, 1985; Jagannathan & Marcotrigiano, 1986; Berlyn *et al.*, 1987). Disease-resistant clones or plants of *Prunus persica* and hybrid *Populus* have been selected by using callus cultured in medium containing phytotoxins (Ettinger *et al.*, 1986; Hammerschalg, 1986). Destructive mycoplasma-like organism have been be eliminated from callus cultures of some crops (Ulrychova & Petru, 1975; Fedotina & Krylova, 1976; Jacoli, 1978; Moller & Sarkar, 1989). Serial subculture of organogenetic and embryogenic callus have been reported in *P.tomentosa* and *P.taiwaniana* (Fan & Hu, 1976; Fu, 1978; Radojevic, 1979). However, attempts to produce somatic embryogenic callus failed in our experiments (Chapter 5) and organogenetic callus of *P.taiwaniana* gradually lost the ability to regenerate when serial subcultured (Fan & Hu, 1976). The aim of this

study was to asses the regenerability of organogenetic and friable callus of *Paulownia* to regenerate, and to provide material for cell suspension cultures.

2. MATERIAL AND METHODS

2.1 Establishment and subculture of organogenetic calli in NAA and BA media

Organogenetic callus derived from internodal explants of four *Paulownia* species cultured in MS medium with 0.5 mg l^{-1} NAA plus 5 mg l^{-1} BA (code O4 medium, the best shoot formation medium reported in Chapter 3) were subcultured on different media. The media used were MS medium (Murashige & Skoog, 1962) solidified with 0.2 % gelrite and supplemented with 0.1 mg l^{-1} NAA and 1 mg l^{-1} BA, or 0.25 mg l^{-1} NAA and 2.5 mg l^{-1} BA, or 0.5 mg l^{-1} and 5 mg l^{-1} BA.

Organogenetic calli derived from shoot tip, nodal, internodal and leaf explants of *P.kawakamii* cultured on MS medium containing combinations of NAA (0.5, 1.0, 2.5 mg l^{-1}) and BA (0.5, 1.0, 2.5, 5.0 mg l^{-1}) solidified with 0.2 % gelrite (media codes: O1-O12 in Chapter 3) were subcultured. Culture media were shoot formation medium (MS medium containing 0.5 mg l^{-1} NAA plus 5 mg l^{-1} BA) or MS medium containing combinations of NAA (0, 0.1 mg l^{-1}) and BA (1, 5, 10 mg l^{-1}).

After removing adventitious buds from the calli, two calli pieces (ca 3 mm^3 in size) were placed into a test tube containing 5 ml media. Treatments were repeated five times. After 6 weeks in culture, the number of adventitious buds and the size of callus were recorded. The callus size was scored as small (+), medium (++), and large (+++). The percentage of calli which turned brown in colour (browning %) was also recorded.

2.2 Establishment and regeneration of friable callus

2.2.1 Induction of friable callus with auxins

Internodal explants of four species of *Paulownia* were prepared as described in Chapter 3. Two internodal explants were placed into a test tube containing 5 ml MS medium supplemented with 0.1, 0.5, 1.0 mg l^{-1} 2,4-D or 0.5, 1.0, 2.5 mg l^{-1} NAA. Treatments were repeated five times. After 6 weeks in culture, callus weight (mg/internodal explant) and browning percentage were recorded.

2.2.2 Induction and subculture of friable callus in 2,4-D and kinetin

Explants used consisted of cotyledons divided in two halves through the centre, hypocotyls and shoot tips as described in Chapter 3 and roots cut into 2-3 mm long sections. Four species of *Paulownia* were used. Two shoot tip, four cotyledon, three hypocotyl and three root explants were used respectively per test tube. Each test tube contained 5 ml MS medium, 3 % sucrose, 2 % gelrite and combinations of 2,4-D (0.001, 0.01, 0.1, 1.0, 2.0 mg l^{-1}) and kinetin (0.1, 1.0 mg l^{-1}).

Calli derived from different explants of *P.kawakamii* and *P.taiwaniana* cultured on MS medium containing 2,4-D and kinetin were subcultured in MS medium solidified with 0.2 % gelrite and containing NAA (0.01, 0.1 mg l^{-1}) and BA (1, 3, 5, 10 mg l^{-1}). Two callus pieces (ca 3 mm^3) were used per test tube containing 5 ml media.

Treatments were repeated five times. After 6 weeks in culture, the number of adventitious buds and roots, callus types, size (represented as -, +, ++, +++ for none, small, medium and large), and browning percentage were recorded.

2.3 Effect of antioxidants on browning of callus

Organogenetic calli derived from internodal explants of four species of *Paulownia* cultured on MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA were subcultured on the same medium. Calli without adventitious buds were cut from the explants under sterile water, or solutions containing different antioxidants. Callus pieces were kept submersed for 3 min. Calli with or without adventitious buds cut from the explants in air served as controls. The concentration of antioxidants used were : 0.1 g l^{-1} cystein·HCl, 1 g l^{-1} ascorbic acid, 1 g l^{-1} ascorbic acid plus 1 g l^{-1} citric acid, 2 g l^{-1} PVP K30 (molecular weight 40,000), 2 g l^{-1} Polyclar, 50 mg l^{-1} DTT, 50 mg l^{-1} thiourea. Two calli pieces (ca 3 mm^3) were used per test tube containing 5 ml medium.

Calli derived from different explants of *P.kawakamii* and *P.taiwaniana* cultured on MS medium containing 2,4-D and kinetin were cut into 3 mm^3 pieces and subcultured on MS medium, MS medium containing 0.01 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin, or MS medium containing the same concentration of 2,4-D and kinetin with 0.01 g l^{-1} citric acid or 3 g l^{-1} activated charcoal. Two pieces of callus were placed in each test tube. Treatments were repeated five times. After 6 weeks in culture, browning percentage of callus was recorded.

In this series of experiments the pH of media was adjusted to 5.7 and then autoclaved for 15 min at a pressure of 1.2 Kg cm^{-2}. Cultures were kept in a culture room with 16 hour photoperiod at 45-60 $\mu Em^{-2}s^{-1}$ fluorescent and incandescent light at 25^{o}C .

3 RESULTS AND DISCUSSION

3.1 Shoot formation on subcultures of organogenetic calli derived from NAA and BA combination

Internodal calli of all four species of *Paulownia* failed to form adventitious buds when subcultured on media containing combinations of NAA and BA (Table 6.1). Irrespective of the media and explant type from which organogenetic calli were derived, adventitious buds failed to form when calli of *P.kawakamii* were subcultured to MS medium plus 0.5 mg l^{-1} NAA and 5 mg l-1 BA) (Tables 6.2.1 - 6.2.4). Most calli remained compact and green to white in colour and were comparable in appearance before calli were subcultured. Calli of *P.kawakamii* subcultured in media containing combinations of NAA (0, 0.1 mg l^{-1}) and BA (1, 5, 10 mg l^{-1}) also failed to form adventitious shoots (Tables 6.3.1 - 6.3.4), except for nodal calli derived from MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA. Most of the calli subcultured on low levels of NAA and high levels of BA turned brown. There was a tendency towards more browning when callus was subcultured on low levels NAA as compared to calli subcultured on shoot forming medium (0.5 mg l^{-1} NAA and 5 mg l^{-1} BA) (Tables 6.2 and 6.3). The loss of regeneration ability of the calli may be related to ploidy changes of the cells when subcultured as reported before (Murashige & Nakano, 1966 and 1967; Smith & Street, 1974; Negrutiu *et al.*, 1975; Chandler *et al.*, 1982).

Table 6.1. Effect of NAA and BA on adventitious bud formation and colour of subcultured callus. Callus was derived from internodal explants of four *Paulownia* species cultured on MS medium plus 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA

NAA mg/l	BA	*fortunei*		*kawakamii*		*tomentosa*		*taiwaniana*	
		AB[1]	Calli	AB	Calli	AB	Calli	AB	Calli
0.1	1.0	0.2	green	0	white	0.4	white	0.4	green
0.25	2.5	0.3	green	0	white	0.8	white	0.8	green
0.5	5.0	0.8	white	0	white	0.2	white	0.5	white

[1]AB: adventitious buds per callus explant

Table 6.2. Effect of shoot formation medium (O4: MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA) on adventitious bud formation and browning of *P.kawakamii* calli derived from different explants cultured on NAA plus BA (medium code O1-O12).

Table 6.2.1. Effect on shoot tip calli

	Shoot tip calli derived from									
	O1	O2	O3	O4	O5	O6	O7	O8	O9	O12
Callus colour	brown	green	green	green	white	brown	brown	green	white	green
Callus growth	-	+++	+	+	+	-	-	+++	++	+++
AB[1]	0	0	0	0.5	0	0	0	0	0	0
Brn%[2]	100	0	0	25	0	100	100	0	0	0

O1-O4 medium: 0.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O5-O8 medium: 1 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O9-O12 medium: 2.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
-, +, ++, +++ represent: none, small, medium and large callus
[1]AB: adventitious buds per callus explants, [2]Brn%: browning %

Table 6.2.2. Effect on nodal calli

	Nodal calli derived from						
	O2	O3	O4	O5	O7	O8	O10
Callus colour	grey	grey	white	green	white	green	green
Callus growth	+	+	+	+	+	++	+
AB[1]	0	0	1	0	0	0	0
Brn%[2]	0	50	0	40	0	40	67

O2-O4 medium: 0.5 mg l^{-1} NAA + 1, 2.5, 5 mg l^{-1} BA
O5-O8 medium: 1.0 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O10 medium: 2.5 mg l^{-1} NAA + 1 mg l^{-1} BA
-, +, ++, +++ represent: none, small, medium and large callus
[1]AB: adventitious buds per callus explants, [2]Brn%: browning %

Table 6.2.3. Effect on internodal calli

	Internodal calli derived from								
	O1	O2	O3	O4	O6	O7	O8	O9	O10
Callus colour	green	brown	green	white	white	brown	brown	green	green
Callus growth	+++	-	+	++	++	-	-	+	+
AB[1]	4.3	0	0.5	0	0	0	0	0	0
Brn%[2]	0	100	25	75	22	100	100	33	60

O1-O4 medium: 0.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O5-O8 medium: 1 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O9-O12 medium: 2.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
-, +, ++, +++ represent: none, small, medium and large callus
[1]AB: adventitious buds per callus explants, [2]Brn%: browning %

Table 6.2.4. Effect on leaf calli

	Leaf calli derived from					
	O2	O3	O4	O7	O8	O9
Callus colour	green	brown	green	brown	brown	white
Callus growth	+	-	+++	-	-	++
AB[1]	0.5	0	0	0	0	0
Brn%[2]	75	100	75	0	0	75

O2-O4 medium: 0.5 mg l^{-1} NAA + 1, 2.5, 5 mg l^{-1} BA
O7-O8 medium: 1 mg l^{-1} NAA + 2.5, 5 mg l^{-1} BA
O9 medium: 2.5 mg l^{-1} NAA + 0.5 mg l^{-1} BA
-, +, ++, +++ represent: none, small, medium and large callus
[1]AB: adventitious buds per callus explants, [2]Brn%: browning %

Table 6.3. Effect of NAA and BA on adventitious bud formation and browning of calli derived from different explants of *P kawakamii* cultured on NAA plus BA medium (medium code: 01-012)

Table 6.3.1 Effect on shoot tip calli

NAA mg l^{-1}	BA mg l^{-1}	Shoot tip calli derived from									
		O1		O3		O4		O6		O7	
		AB[1]	Brn%[2]	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%
0	1	0	100	0	100	0	100	0	100	0	100
0	5	0	100	3.5	25	0	100	0	100	0	100
0	10	0	100	0.5	30	0	100	0	100	0	80
0.1	1	0	100	0	20	0	100	0	100	0	20
0.1	5	0	100	0	100	2	50	0	50	0	80
0.1	10	0	100	0	25	0	100	0	50	1	20

O1-O4 medium: 0.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O6-O7 medium: 1 mg l^{-1} NAA + 1, 2.5 mg l^{-1} BA
[1]AB: adventitious buds per callus explant, [2]Brn%: Browning %

Table 6.3.2. Effect on nodal calli

NAA mg l^{-1}	BA mg l^{-1}	Nodal calli derived from													
		O2		O3		O4		O7		O8		O9		O12	
		AB[1]	Brn%[2]	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%
0	1	0	100	0	100	8	20	0	100	1	90	0	100	0	100
0	5	0	100	0	100	4	20	0	100	1	90	0	100	0	100
0	10	0	100	0	100	1	60	0	100	1	90	0	80	0	100
0.1	1	0	100	0	100	0	100	2	80	0	100	0	80	0	100
0.1	5	0	100	0	100	0	100	0	100	0	100	0	40	0	100
0.1	10	0	100	0	100	0	100	0	100	0	100	0	100	0	100

O2-O4 medium: 0.5 mg l^{-1} NAA + 1, 2.5, 5 mg l^{-1} BA
O7-O8 medium: 1 mg l^{-1} NAA + 2.5, 5 mg l^{-1} BA
O9,O12 medium: 2.5 mg l^{-1} NAA + 0.5, 5 mg l^{-1} BA
[1]AB: adventitious buds per callus explant, [2]Brn%: Browning %

Table 6.3.3. Effect on internodal calli

NAA mg l^{-1}	BA mg l^{-1}	Internodal calli derived from													
		O1		O2		O4		O6		O8		O9		O10	
		AB[1]	Brn%[2]	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%
0	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100
0	5	0	100	0	100	0	100	2	20	0	80	0	100	0	100
0	10	0	100	0	50	0	100	0	100	0	20	0	100	0	100
0.1	1	0	100	0	100	0	100	0	80	0	20	0	100	0	100
0.1	5	0	100	0	100	0	100	0	100	0	80	0	100	0	100
0.1	10	0	100	0	50	0	50	0	100	0	100	0	100	0	100

O1-O4 medium: 0.5 mg l^{-1} NAA + 0.5, 1, 2.5, 5 mg l^{-1} BA
O6,O8 medium: 1 mg l^{-1} NAA + 1, 5 mg l^{-1} BA
O9-10 medium: 2.5 mg l^{-1} NAA + 0.5, 1 mg l^{-1} BA
[1]AB: adventitious buds per callus explant, [2]Brn%: Browning %

Table 6.3.4. Effect on leaf calli

NAA mg l^{-1}	BA	Leaf calli derived from									
		O2		O3		O4		O7		O8	
		AB[1]	Brn%[2]	AB	Brn%	AB	Brn%	AB	Brn%	AB	Brn%
0	1	0	100	0	100	0	100	0	100	0	100
0	5	0	100	0	100	0	60	0.3	20	0	100
0	10	0	100	0	100	0	20	0	50	0	100
0.1	1	0	100	0	100	0	100	0	20	0	100
0.1	5	0	100	0	100	0	20	0.3	20	0	100
0.1	10	0	100	0	100	0	100	0	100	1	100

O2-O4 medium: 0.5 mg l^{-1} NAA + 1, 2.5, 5 mg l^{-1} BA
O7-O8 medium: 1 mg l^{-1} NAA + 2.5, 5 mg l^{-1} BA
[1]AB: adventitious buds per callus explant, [2]Brn%: Browning %

3.2 Effect of auxins and kinetin on callus growth and organogenesis

Large friable callus growth was induced by 0.1 mg l^{-1} 2,4-D in the case of *P.kawakamii* and *P.tomentosa* (Table 6.4). High levels of 2,4-D caused explants to turn brown in colour (Table 6.5). NAA induced small compact callus and many roots formed. Increased NAA concentrations enhanced root formation for *P.fortunei* and *P.kawakamii*, increased root numbers of *P.tomentosa*, and roots formed less readily with *P.taiwaniana*. Except for *P.tomentosa*, calli of all *Paulownia* species turned brown when NAA at 2.5 mg l^{-1} was used.

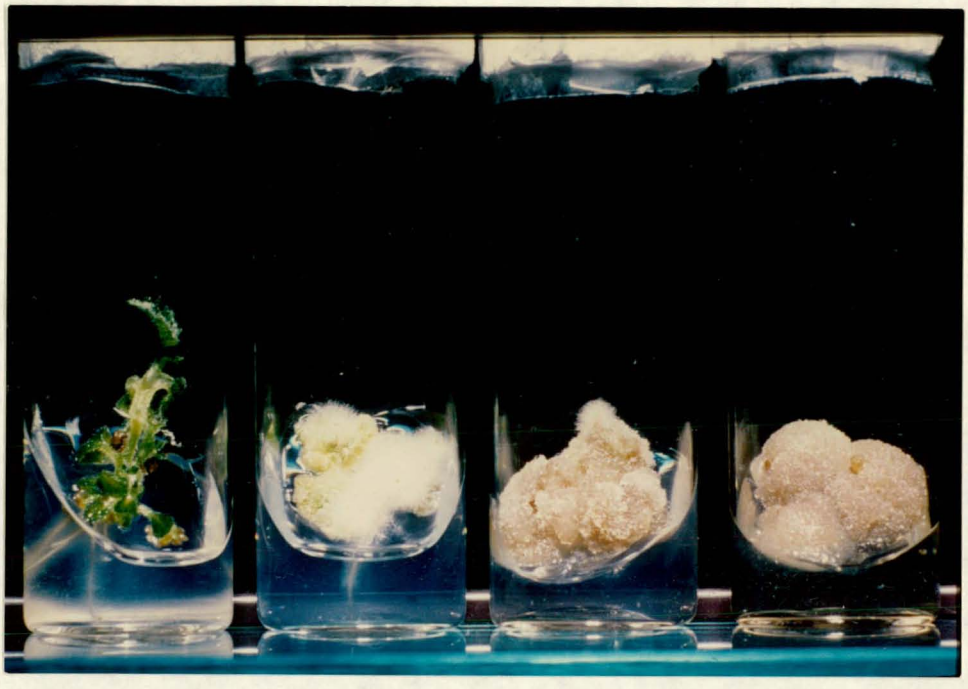
Combinations of 2,4-D and kinetin caused more variation in callus types (Tables 6.6 - 6.9). In general four distinctive types of calli could be identified depending on the level of 2,4-D used. Small compact calli green in colour formed in 0.001 mg l^{-1} 2,4-D, small to medium in size, compact and dish-shaped callus, green in colour formed in 0.01 mg l^{-1} 2,4-D, large sized friable callus white or white grey in colour formed in 0.1 mg l^{-1} 2,4-D and small to medium sized calli grey or brown in colour formed in 1.0 mg l^{-1} 2,4-D (Fig. 6.1). Adventitious buds formed in callus tissues in all *Paulownia* species except *P.tomentosa*. Few adventitious buds formed from shoot tip explants in low levels of 2,4-D and 1 mg l^{-1} kinetin for *P.fortunei* and *P.taiwaniana* while for *P.kawakamii* more adventitious buds formed from shoot tip and hypocotyl explants in the same range of 2,4-D. Roots formed more readily in shoot tip explants than in other explants for all *Paulownia* species. Increasing kinetin concentration reduced root formation. Compared to the organogenesis in NAA and BA combinations (Chapter 3) it appeared that *P.kawakamii* has a higher organogenetic potential in a wide range of auxins and cytokinins than for the other three species. When 2,4-D concentration increased up to 2 mg l^{-1}, all explants turned brown and died (data not shown).

Table 6.4. Effect of auxins on callus growth (fresh weight) of internodal explants for *Paulownia* species

Auxins mg l^{-1}	Callus weight (mg per explant)			
	fortunei	*kawkamii*	*tomentosa*	*taiwaniana*
2,4-D				
0.1	196±8.2	447±105.6	729±208.8	166±56.3
0.5	-	-	42±7.2	-
1.0	-	-	37±7.8	-
NAA				
0.5	73±19.1	170±34.9	221±69.8	51±18.2
1.0	98±21.3	112±30.7	41±6.7	-
2.5	92±16.9	72±6.4	326±72.8	-

Table 6.5. Effect of auxins on root formation and browning of internodal explants for *Paulownia* species.

Auxins mg l^{-1}	*fortunei*		*kawkamii*		*tomentosa*		*taiwaniana*	
	Roots per explant	Brown %	Roots per explant	Brown %	Roots per explant	Brown %	Roots per explant	Brown %
2,4-D								
0.1	0	0	0	0	6.3	0	1.3	0
0.5	0	80	0	100	0	40	0	100
1.0	0	100	0	100	0	40	0	100
NAA								
0.5	1.7	0	1	20	3.6	20	0.5	0
1.0	2.5	40	1.8	0	4.4	20	0.5	40
2.5	2.3	60	3.4	60	0	20	0	80



2,4-D	0.001	0.01	0.1	1.0 mg l^{-1}
kinetin	1.0	1.0	0.1	0.1

Fig. 6.1. Morphogenesis in cotyledon explants of P.kawakamii cultured on MS medium containing 2,4-D and kinetin. (a) formation of vitrified adventitious buds, (b) compact green callus dish-like in shape , (c) friable callus, grey white in colour, (d) grey callus. Bar represents 1 cm.

Table 6.6. Effect of combinations of 2,4-D and kinetin on callus formation and organogenesis from different explants of *P.fortunei*

Table 6.6.1. Effect on callus formation

2,4-D mg l^{-1}	Kin	Shoot tips		Cotyledons		Hypocotyls		Roots	
		Colour	Size	Colour	Size	Colour	Size	Colour	Size
0.001	0.1	green	+	brown	-	green	+	-	-
0.01	0.1	green	+	green	+	green	+	-	-
0.1	0.1	grywht[1]	+	green	++	green	+++	grywht	+
1.0	0.1	grey	+++	grywht	++	grey	+	grey	+
0.001	1.0	green	+	green	+	green	+	green	+
0.01	1.0	grywht	++	green	++	green	++	green	++
0.1	1.0	grywht	+++	green	+++	grnwht[2]	+++	grnwht	++
1.0	1.0	grey	+++	grywht	+++	grey	+	grey	+

[1]grywht: grey and white, [2]grnwht: green and white,
-, +, ++, +++ represent: none, small, medium and large callus

Table 6.6.2. Effect on organogenesis

2,4-D (mg l^{-1})	Kin	Shoot tips		Cotyledons	Hypocotyls	Roots
		AB/exp[1]	Rt/exp[2]	Rt/exp	Rt/exp	Rt/exp
0.001	0.1	0	2.0±0.7	0	1.3±0.6	1.0±0.8
0.01	0.1	0	2.8±0.4	1.3±0.5	2.3±0.6	4.0±0.6
0.1	0.1	1.0±0.8	2.0±0.7	3.1±0.6	6.0±2.5	3.9±1.1
1.0	0.1	0	3.3±1.1	1.0±0.8	0	0
0.001	1.0	2.0±1.1	2.3±0.6	0.3±0.2	0	0.1±0.1
0.01	1.0	1.0±0.5	3.3±0.7	1.7±0.8	0	2.0±0.8
0.1	1.0	0	4.8±0.9	3.7±0.6	3.0±1.3	0.9±0.7
1.0	1.0	0	1.3±1.0	0	0	0

[1]AB/exp: Adventitious buds per explant, [2]Rt/exp: Roots per explant

Table 6.7. Effect of combinations of 2,4-D and kinetin on callus formation and organogenesis from different explants of *P.kawakamii*

Table 6.7.1 Effect on callus formation

2,4-D mg l^{-1}	Kin	Shoot tips		Cotyledons		Hypocotyls		Roots	
		Colour	Size	Colour	Size	Colour	Size	Colour	Size
0.001	0.1	-	+	brown	-	green	+	brown	-
0.01	0.1	green	+	green	++	green	+	green	+
0.1	0.1	grywht[1]	+++	grywht	+++	grywht	+++	grywht	+++
1.0	0.1	grey	+++	grey	+++	grey	+	grey	+
0.001	1.0	green	+	green	+	green	+	green	+
0.01	1.0	green	++	green	+++	green	++	green	++
0.1	1.0	grywht	+++	green	+++	grnwht[2]	+++	grnwht	++
1.0	1.0	grey	+++	grey	+++	brown	-	brown	-

[1]grywht: grey and white, [2]grnwht: green and white
-, +, ++, +++ represent: none, small, medium and large callus

Table 6.7.2. Effect on organogenesis

2,4-D mg l^{-1}	Kin	Shoot tips		Cotyledons		Hypocotyls		Roots
		AB/exp[1]	Rt/exp[2]	AB/exp	Rt/exp	AB/exp	Rt/exp	Rt/exp
0.001	0.1	0	1.2±0.1	0	0	0	0	0
0.01	0.1	0	1.3±0.4	0	3.4±0.4	0	0.6±0.2	0
0.1	0.1	0	0.3±0.2	0	3.4±1.1	0	1.8±0.3	3.3±0.5
1.0	0.1	0	0	0	0	0	0	0
0.001	1.0	12±2.4	0.3±0.2	0.5±0.4	0.1±0.1	4.5±2.8	0	0
0.01	1.0	11±2.4	0.8±0.2	0	0.4±0.2	9.9±2.3	1.1±0.7	1.1±0.1
0.1	1.0	0	1.0±0.5	0	1.0±0.2	0	0	0
1.0	1.0	0	0	0	0	0	0	0

[1]AB/exp: adventitious buds per explant, Rt/exp: roots per explant

Table 6.8. Effect of combinations of 2,4-D and kinetin on callus formation and organogenesis from different explants of *P. tomentosa*

Table 6.8.1. Effect on callus formation

2,4-D mg l^{-1}	Kin	Shoot tips		Cotyledons		Hypocotyls		Roots	
		Colour	Size	Colour	Size	Colour	Size	Colour	Size
0.001	0.1	green	+	green	+	green	+	green	++
0.01	0.1	green	+	green	+	green	+	green	++
0.1	0.1	grywht[1]	++	green	++	green	++	grywht	+
1.0	0.1	grywht	++	grywht	++	grey	+	grey	+
0.001	1.0	green	+	green	+	green	+	green	+
0.01	1.0	grywht	++	green	+	green	+	green	+
0.1	1.0	grnwht[2]	++	green	+	green	+++	green	++
1.0	1.0	grywht	++	grey	+	brown	-	grey	+

[1]grywht: grey and white, [2]grnwht: green and white.
-, +, ++, +++ represent: none, small, medium and large callus

Table 6.8.2. Effect on organogenesis

2,4-D mg l^{-1}	Kin	Shoot tips	Cotyledons	Hypocotyls	Roots
		Rt/exp[1]	Rt/exp	Rt/exp	Rt/exp
0.001	0.1	1.0±0.3	0.1±0.1	0	0.3±0.1
0.01	0.1	1.4±0.2	2.3±0.1	0.8±0.4	1.2±0.3
0.1	0.1	3.4±1.0	2.1±0.0	0.8±0.6	0.3±0.1
1.0	0.1	0.5±0.4	0	0	0
0.001	1.0	0	0.1±0.1	0	0.6±0.3
0.01	1.0	1.2±0.5	1.7±0.4	1.4±0.8	0.8±0.2
0.1	1.0	3.3±0.5	1.4±0.4	2.5±0.2	0.8±0.6
1.0	1.0	0	0	0	0

[1]Rts/exp: roots per explants

Table 6.9. Effect of combinations of 2,4-D and kinetin on callus formation and organogenesis from different explants of *P.taiwaniana*

Table 6.9.1. Effect on callus formation

2,4-D mg l^{-1}	Kin	Shoot tips		Cotyledons		Hypocotyls		Roots	
		Colour	Size	Colour	Size	Colour	Size	Colour	Size
0.001	0.1	-	-	brown	-	-	-	-	-
0.01	0.1	green	+	green	++	-	-	green	+
0.1	0.1	white	+++	white	++	white	++	green	++
1.0	0.1	white	+++	grey	++	brown	-	brown	-
0.001	1.0	green	+	brown	-	green	+	green	+
0.01	1.0	green	+++	green	++	green	++	green	+
0.1	1.0	white	+++	green	++	white	++	white	+++
1.0	1.0	grywht[1]	++	white	+++	grey	+	brown	-

[1]grywht: grey and white.
-, +, ++, +++ represent: none, small, medium and large callus

Table 6.9.2. Effect on organogenesis

2,4-D mg l^{-1}	Kin	Shoot tip		Cotyledon	Hypocotyl	Root
		AB/exp[1]	Rt/exp[2]	Rt/exp	Rt/exp	Rt/exp
0.001	0.1	0	2.7±0.2	0.1±0.2	0	0
0.01	0.1	0	1.7±0.2	0.2±0.1	1.3±0.1	0
0.1	0.1	0	2.3±1.7	1.1±0.1	0	0
1.0	0.1	0	0	0	0	0
0.001	1.0	0	1.0±0.3	0	0	0
0.01	1.0	1.0±0.0	1.5±0.2	1.3±0.6	0	0
0.1	1.0	0	1.3±0.4	1.3±0.4	1.0±0.5	0.6±0.1
1.0	1.0	0	0	0	0	0

[1]AB/exp: adventitious buds per explant, [2]Rt/exp: roots per explant.

3.3 Adventitious bud formation on calli derived from 2,4-D and kinetin combination

Most of *P.kawakamii* calli derived from 2,4-D and kinetin media turned brown when subcultured on NAA and BA media except those derived from 0.01 mg l^{-1} 2,4-D and 0.1 mg $^{-1}$ kinetin (Table 6.10.1). For *P.taiwaniana*, more calli survived when subcultured than was the case with *P.kawakamii* (Table 6.11.1). Subcultured calli of *P.kawakamii* lost their organogenetic ability in NAA and BA media (Table 6.10.2). *P.taiwaniana* calli especially shoot tip calli formed adventitious buds when subcultured in 0.01 mg l^{-1} NAA and 10 mg l^{-1} BA (Table 6.11.2).

Table 6.10. Effect of NAA and BA on callus browning and organogenesis of *P.kawakamii* derived from 2,4-D plus kinetin

Table 6.10.1. Effect on browning (%)

NAA mg l^{-1}	BA	Callus derived from																	
		Shoot tips			Cotyledons						Hypocotyls					Roots			
		DK2	DK3	DK7	DK2	DK3	DK4	DK6	DK7	DK8	DK2	DK3	DK4	DK6	DK7	DK2	DK3	DK6	DK7
0.01	1	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	100	100
0.01	3	100	100	100	100	50	67	100	100	100	0	67	100	100	33	100	100	0	100
0.01	5	100	100	100	0	100	100	100	100	100	0	67	100	100	100	100	100	100	100
0.01	10	100	100	100	100	100	100	100	100	100	100	100	67	100	33	100	100	0	100
0.1	1	0	100	100	0	100	100	100	100	50	0	0	100	100	100	0	100	0	100
0.1	3	100	100	100	0	67	100	100	50	100	0	33	100	0	100	33	50	0	0
0.1	5	50	100	100	0	100	100	100	100	50	0	0	100	100	50	0	50	0	0
0.1	10	0	100	100	0	33	33	100	50	100	100	0	100	100	67	100	50	100	100

DK2-4: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
DK6-8: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin

Table 6.10.2. Effect on organogenesis of calli derived from MS medium plus 0.01 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin (DK_2 medium)

NAA mg l^{-1}	BA	Callus derived from							
		Shoot tips		Cotyledons		Hypocotyls		Roots	
		AB[1]	Rt[2]	AB	Rt	AB	Rt	AB	Rt
0.01	1	-	-	-	-	1	1	-	-
0.01	5	-	-	1	-	1	-	-	-
0.1	1	-	2	-	-	-	-	-	-
0.1	3	-	-	-	-	-	-	-	2

[1]AB: adventitious buds per callus explant, Rt: roots per callus explant

Table 6.11. Effect of NAA and BA on callus browning and organogenesis of *P.taiwaniana* derived from 2,4-D plus kinetin

Table 6.11.1. Effect on browning (%)

NAA mg l^{-1}	BA	Callus derived from												
		Shoot tips					Cotyledons				Hypocotyls			Roots
		DK3	DK4	DK6	DK7	DK8	DK3	DK4	DK7	DK8	DK3	DK6	DK7	DK7
0.01	1	100	0	0	100	0	0	0	100	100	0	100	100	100
0.01	3	100	0	0	100	0	100	0	100	100	0	100	100	100
0.01	5	100	0	0	100	0	100	0	100	100	0	100	100	100
0.01	10	100	50	0	100	0	100	0	100	100	0	100	100	100
0.1	1	100	50	0	100	0	100	0	100	100	0	100	100	0
0.1	3	100	0	0	100	0	100	0	100	0	100	100	0	0
0.1	5	100	50	0	100	0	100	0	100	0	100	100	100	0
0.1	10	100	50	0	100	0	0	50	100	100	0	100	100	0

DK3-4: 0.1, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
DK6-8: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin

Table 6.11.2. Effect on organogenesis

NAA mg l^{-1}	BA	Callus derived from											
		Shoot tip						Cotyledon				Root			
		DK4		DK6		DK8		DK3		DK4		DK8		DK7	
		AB[1]	Rt[2]	AB	Rt	AB	Rt	AB	Rt	AB	Rt	AB	Rt	AB	Rt
0.01	1	-	4	1	-	-	-	-	11	-	-	-	-	-	-
0.01	3	-	6	6	-	-	5	-	-	-	6	-	-	-	-
0.01	5	-	5	1	-	-	2	-	-	-	3	-	-	-	-
0.01	10	-	5	35	-	-	10	-	-	-	1	-	-	-	-
0.1	1	-	7	-	-	-	0	-	-	-	5	-	-	-	-
0.1	3	-	10	-	-	-	0	-	-	-	2	-	1	-	1
0.1	5	-	-	-	-	-	12	-	-	-	12	-	-	-	-
0.1	10	-	2	-	-	-	1	-	-	-	-	-	-	-	-

[1]AB: adventitious buds per callus explant, [2]RT: roots per callus explant.
DK3-4: 0.1, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
DK6-8: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin

3.4 Effect of antioxidants on callus browning

Soaking calli in antioxidants or water did not reduce browning of *P.kawakamii*, *P.tomentosa* and *P.taiwaniana*. Browning was enhanced when compared to untreated calli (Table 6.12). *P.kawakamii* turned brown more readily than the calli of the other species. Ascorbic acid and ascorbic acid plus citric acid caused a slight reduction in browning of *P.fortunei* calli while PVP K30, thiourea, cystein and DTT increased the incidence of browning. Antioxidants are frequently used to prevent or reduce the oxidation of phenolic compounds (Lee & Skoog, 1965; Loomis & Battaile, 1966; Anderson, 1975; De Forssard & Bourne, 1976; Ichihashi & Kake, 1977; Erez, 1978; Mayer & Harel, 1979; Gupta *et al.*, 1980; Bonga, 1981; George & Sherrington, 1984; Vaughn & Duke, 1984; Compton & Preece, 1986). Phenols may promote callus growth and adventitious bud formation (Compton & Preece, 1988). The reduction in calli browning when calli were not soaked in solutions indicate that growth factors such as some promotive phenols, K^+ and adenine nucleotide may be lost from the tissues during soaking in solution (Gronewald & Hanson, 1980; Compton & Preece, 1988).

Calli of *P.kawakamii* derived from MS medium containing 2,4-D and kinetin medium subcultured on MS medium without hormones turned brown (Tables 6.13.1 and 13.2). Calli derived from MS medium plus 0.01 mg l^{-1} 2,4-D and 1.0 mg l^{-1} kinetin subcultured on the same medium did not turn brown and kept the original compact structure green in colour. Citric acid included into the medium did not reduce callus browning. But the structure of the callus changed from compact green in colour to friable white. Activated charcoal increased browning. This may be due to the absorption of hormones, organic or inorganic compounds which could affect callus growth negatively (Proskauer & Berman, 1970; Klein & Bopp, 1971; Wang & Huang, 1976; Wernick & Kohlenbach, 1976; Compton & Preece, 1986; Bon *et al.*, 1988).

Table 6.12. Effect of different antioxidants on the browning percentages of subcultured organogenetic callus derived from MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA for four *Paulownia* species.

Antioxidants	Browning % of callus			
	fortunei	*kawakamii*	*tomentosa*	*taiwaniana*
Cystein	60	100	100	20
Ascorbic acid	20	100	100	100
Ascorbic acid + Citric acid	0	100	70	90
PVP K30	100	100	100	70
Polyclar	40	100	100	90
DTT	80	100	100	70
Thiourea	60	100	100	80
Water	45	100	100	100
Control				
Callus	40	80	20	40
Callus + AB[1]	0	20	20	20

[1]Callus + AB: callus containing adventitious buds

Table 6.13. Effect of MS medium alone and MS medium containing 0.01 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin with or without 0.01 g l^{-1} citric acid and with 3 g l_{-1} activated charcoal on the browning % on the subculture callus from DK medium for *P.kawakamii*

Table 6.13.1. Calli derived from shoot tip and cotyledon explants

2,4-D mg l^{-1}	Kin	Addenda	Shoot tips						Cotyledons				
			DK3	DK4	DK6	DK7	DK8	DK3	DK4	DK6	DK7	DK8	DK3
0	0	-	100	100	100	100	100	100	100	100	100	100	100
0.01	1	-	0	75	0	100	100	75	100	0	50	100	100
0.01	1	Citric[1]	0	75	0	25	75	75	100	0	100	100	25
0.01	1	Charc[2]	100	100	100	100	75	100	100	100	100	100	100

DK3-4: 0.1, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
DK6-8: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin
[1]Citric: citric acid, [2]Charc: activated charcoal

Table 6.13.2. Calli derived from hypocotyl and root explants

2,4-D mg l^{-1}	Kin mg l^{-1}	Addenda	Hypocotyls			Roots		
			DK4	DK6	DK7	DK3	DK6	DK7
0	0	-	100	100	100	100	100	100
0.01	1	-	100	0	0	100	0	100
0.01	1	Citric[1]	100	0	0	100	0	0
0.01	1	Charc[2]	100	83	100	100	0	100

DK3-4: 0.1, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
DK6-8: 0.01, 0.1, 1.0 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin
[1]Citric: citric acid, [2]Charc: activated charcoal

4. References

Anderson, W.C., 1975. Propagation of Rhododendrons by tissue culture: Part I, development of culture medium for multiplication of shoots. Comb. Proc. Int. Plant Prop. Soc. 25:129-135

Bajaj, Y.P.S., 1990. Somaclonal variation - origin, induction, cryopreservation, and implications in plant breeding. In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry 11: Somaclonal variation in crop improvement I. (pp. 3-48) Springer-Verlag Berlin

Berlyn, G.P., Anoruo, A.O., Beck, R.C. and Cheng, J., 1987. DNA content polymorphism and tissue culture regeneration in Caribbean pine. Can. J. Bot. 65:954-961

Bon, M.C., Gendraud, M. and Franclet, A., 1988. Roles of phenolic compounds on micropropagation of juvenile and mature clones of *Sequoiadendron giganteum*: Influence of activated charcoal. SciHort. 34:283-291

Bonga, J.M., 1981. Organogenesis *in vitro* of tissues from mature conifers. In Vitro 17(6):511-518

Chandler, S.F., Dodds, J.H. and Henshaw, G.G., 1982. Factors affecting adventitious shoot formation in *Solanum laciniatum* callus cultures. In: Fujiwara, A. (Ed.) Plant tissue cultures (pp. 133-134) Maruzen, Tokyo

Compton, M.E. and Preece, J.E., 1986. Exudation and explant establishment. Newsl. Int. Assoc. Plant Tissue Cult. 50:9-18

Compton, M.E. and Preece, J.E., 1988. Response of tobacco callus to shoot tip exudation from five species. Hort.Sci. 23(1):208-210

De Fossard, R.A. and Bourne, R.A., 1976. Vegetative propagation of *Eucalyptus ficifolia* F. Muell by nodal culture *in vitro*. Proc. Int. Plant Prop. Soc. 26:373-378

Erez, A., 1978. Thiourea, a growth promoter of callus tissues. J. Exp. Bot. 29:159-165

Ettinger, T.L., Ostry, M.E., Hackett, W.P., Read, P.E. and Skilling, D.D., 1986. Utilization of somaclonal variation in the development of *Septoria musiva*-resistant hybrid *Populus*. 6th Int. Congr. Plant tissue & Cell Cult. Abstr. Univ. Minnesota Minneapolis (p. 214)

Fan, M.L. and Hu, T.W., 1976. Plantlets from *Paulownia* tissue cultures. Bull. of Taiwan For. Res. Inst. No. 286:1-6

Fedotina, V.L. and Krylova, N.V., 1976. Ridding tobaccos of the mycoplasmic infection big bud by the method of tissue culturing. Dokl. Bot. Sci. 228:48-51

Fu, M.L., 1978. Plantlets from *Paulownia* tissue culture. Gardens Bull. (Singapore) 31:61-66

George, E.F. and Sherrington, P.D., 1984. Plant propagation by tissue culture. Handbook and Directory of Commercial Laboratories. Exegetics Ltd. England

Gronewald, J.W. and Hanson, J.B., 1980. Sensitivity of the proton and ion transport mechanisms of corn roots to injury. Plant Sci. Lett. 18:143-150

Gupta, P.K., Nadgir, A.L., Mascarenhas, A.F. and Jagannathan, V., 1980. Tissue culture of forest trees: clonal multiplication of *Tectona grandis* L. (Teak) by tissue culture. Plant Sci. Lett. 17:259-268

Hammerschalg, F., 1986. *In vitro* selection of peach cells for insensitivity to a toxin produced by *Xanthomonas campestris* cv. Pruni. In: 6th Int. Cong. Plant Tissue & Cell Cult. Abstr. Univ. Minnesota. Minneapolis.(p. 74)

Ichihashi, S. and Kake, S., 1977. Studies on clonal propagation of *Cattleya* through tissue culture methods II: Browning of *Cattleya*. J. Jap. Soc. Hort. Sci. 46:325-350

Jacoli, G.G., 1978. Sequential degeneration of mycoplasma like bodies in plant tissue cultures infected with aster yellows. Can. J. Bot. 56:133-140

Jagannathan, L. and Marcotrigiano, M., 1986. Phenotypic and ploidy status of *Paulownia tomentosa* trees regenerated from cultured hypocotyls. Plant Cell, Tissue & Organ Cult. 7:227-236

Keese, R.J., Rupert, E.A. and Carter Jr., G.E., 1991. Investigations of proliferative and senescent callus of soybean. Physiol. Plant. 81:512-517

Klein, B. and Bopp, M., 1971. Effect of activated charcoal in agar on the culture of lower plants. Nature 230:474

Lee, T.T. and Skoog, F., 1965. Effects of substituted phenols on bud formation and growth of tobacco tissue cultures. Physiol. Plant. 18:387-401

Loomis, W.D. and Battaile, J., 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochem. 5:423-438

Mayer, A.N. and Harel, E., 1979. Polyphenol oxidases in plants. Phytochem. 18:193-215

Moller, C. and Sarkar, S., 1989. Regeneration of healthy plants from *Catharanthus roseus* infected with mycoplasma-like organisms through callus culture. Plant Sci. 60:83-89

Murashige, T. and Nakano, R., 1966. Tissue culture as a potential tool in obtaining polyploid plants. J. Hered. 57:115-118

Murashige, T. and Nakano, R., 1967. Chromosome complement as a determinant of the morphogenic potential of tobacco cells. Ann. J. Bot. 54:963-970

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-479

Negrutiu, I., Beeftink, F. and Jacobs, M., 1975. *Arabidopsis thaliana* as a model system in somatic cell genetics I: Cell and tissue culture. Plant Sci. Lett. 5:293-304

Ogura, H., 1990. Chromosome variation in plant tissue culture. In: Bajaj, Y.P.S. (Ed.) Biotechnology in agriculture and forestry 11: Somaclonal variation in crop improvement I. (pp. 49-84) Springer-Verlag Berlin

Proskauer, J. and Berman, R., 1970. Agar culture medium modified to approximate soil conditions. Nature 227:1161

Radojevic, L., 1979. Somatic embryos and plantlets from callus cultures of *Paulownia tomentosa* Steud. Z. Pflanzenphysiol. 91:57-62

Renfroe, M.H. and Berlyn, G.P., 1985. Variation in nuclear DNA content in *Pinus taeda* L. tissue culture of diploid origin. J. Plant Physiol. 121:131-139

Smith, S.M. and Street, H.E., 1974. The decline of embryogenic potential as callus and suspension culture of carrot (*Daucus carota* L.) are serially subcultured. Ann. Bot. 38:223-241

Ulrychova, M. and Petru E., 1975. Elimination of mycoplasma in tobacco callus tissues (*Nicotiana gluca* Grah) cultured in the presence of 2,4-D in the nutrient medium. Biol. Plant. 17:103-108

Vaughn, K.C. and Duke, S.O., 1984. Function of polyphenol oxidase in higher plants. Physiol. Plant. 60:106-112

Wang, P.J. and Huang, L.C., 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. In Vitro 12:260-262

CHAPTER 7

CELL SUSPENSIONS

Key words: cell suspension, cell culture, *Paulownia* species

ABSTRACT

Cell suspensions of *Paulownia tomentosa* were obtained from friable callus which was obtained by culturing shoot tip explants on MS medium containing 2.5 mg l^{-1} NAA and BA. Cell suspension of *P.tomentosa* and *P.taiwaniana* were also obtained without callus interphase by culturing seedling shoots. MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin were used. Cultures were kept in the dark on a gyratory shaker at 100 rpm. Cell densities of 4.5 x 10^5 were achieved by using 200 mg explant. Most cells (75 - 95 %) were viable and cell suspension of *P.tomentosa* and *P.taiwaniana* had a high growth rate. The cultures were maintained for more than one year by regular subculturing. The same medium was used to subculture *P.tomentosa* cells, but for successful subculture of *P.taiwaniana* 2,4-D and kinetin concentration was decreased and NAA and BA was necessary. This method of establishing cell suspension culture is effective and time-saving compared to the generally accepted method using callus tissue. The production of friable callus on agar suitable for suspension culture is eliminated. Callus formation from suspension cells for both species wase achieved by cell plating or transferring suspension cells into liquid MS medium containing NAA and BA. Organogenesis from callus was, however, not achieved.

ABBREVIATIONS USED

BA = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; FDA = Fluorescein diacetate; NAA = α-naphthaleneacetic acid, OD = Optical density

1. INTRODUCTION

Cell suspension cultures are useful to generate rapidly large quantities of cell material from which homogeneous cell populations can be sampled for studies on the regulation of plant cell metabolism, cell growth and division (King *et al.*, 1973; King, 1980). Most of the studies in this area have been performed with herbaceous and crop plants. There are only a few reports in the literature of successful cell suspensions of woody plants. Suspension cultures of woody plants are more difficult to establish (Teulières *et al.*, 1989). Objectives of earlier research included mass cell generation, protoplast isolation, cell fusion and gene transformation (Winton, 1968; Hurwitz & Agrios, 1984; Tremblay *et al.*, 1985; Hustache *et al.*, 1986; Matsuta *et al.*, 1986; Attree *et al.*, 1987; Bekkaoui, *et al.*, 1987; Park & Son, 1988; Tremblay, 1988; Cheema, 1989; Finer *et al.*, 1989; Huang *et al.*, 1990; Kumar *et al.*, 1991; Touchet *et al.*, 1991), to select frost tolerant cell lines (Hellergren, 1983; Teulieres *et al.*, 1989), to study cell growth, metabolism and nucleotide composition (Henshaw *et al.*, 1966; Givan & Collin, 1967; Brown & Short, 1969; King *et al.*, 1973, 1974; Wilson & Street, 1975; Bernard *et al.*, 1979; Carrier *et al.*, 1990; Messner *et al.*, 1991; Tate & Payne, 1991) and to produce secondary plant products (Orihara & Furuya, 1990; Carrier *et al.*, 1991). Establishment and maintaining cell suspensions have not been reported for *Paulownia*. This study reports on the successful establishment and maintenance of cell suspensions

of *P.tomentosa* and *P.taiwaniana*. The ability to regenerate cell culture by cell plating or cell aggregates for *Paulownia* species is also reported.

2. MATERIAL AND METHODS

2.1 Suspension culture from callus

Compact callus or friable callus (ca 2-3 g) from shoot tip, cotyledon, hypocotyl and root explants of four *Paulownia* species cultured on MS medium containing 2,4-D and kinetin or NAA and BA as described in Chapter 4 were used in this experiment. Callus tissues were placed in liquid media, which consisted of MS medium containing 0.5 and 1.0 mg l^{-1} 2,4-D, or combinations of NAA (2.5, 5.0 mg l^{-1}) and BA (0.5, 1.0, 2.5 and 5.0 mg l^{-1}). Callus tissues (ca 2-3 g) were cultured in 100 ml Pyrex Erlenmeyer flasks containing 20 ml media. Treatments were repeated 3 times. The cultures were placed on a gyratory shaker at 100 rpm and grown at 25^{o}C in the dark. After 2 weeks in culture, 10 ml aliquots of cell suspension were filtered through a 1000 μm mesh and transferred to new flasks containing 20 ml fresh media. In subsequent subcultures 5 ml of cell suspension culture was taken up in a Pasteur pipette, after allowing the suspension to settle for 1 min, and transferred to a new flask containing 15 ml fresh medium. Subcultures were done when most cells entered the stationary growth phase as determined visually under a inverted microscope. Viable cells were determined using fluorescein diacetate (FDA) (Widholm, 1972).

2.2 Suspension culture from explants

2.2.1 Establishment of tissue culture

Seeds of *P.fortunei*, *P.kawakamii*, *P.taiwaniana* and *P.tomentosa* were sterilized for one min in 70 % ethanol, 12 min in commercial bleach and a drop of Tween 20 per 100 ml and rinsed three times with sterile distilled water. The seeds were placed in 220 ml glass jars containing 30 ml solidified MS basal medium (Murashige & Skoog, 1962) supplemented with 3% sucrose and 0.7% Biolab agar (Biolab Diagnostics Ltd). The pH of the media was adjusted to 5.7 and then autoclaved for 15 min at 1.2 Kg cm^{-2} pressure. Cultures were kept in a culture room at 25°C with a 16 hour photoperiod at 45-60 $\mu Em^{-2}s^{-1}$ fluorescent and incandescent light. Growth was maintained by subculturing shoot tips with 1 to 2 leaves onto fresh MS medium every 1.5 months.

2.2.2 Establishment of cell suspension cultures

The following explants of *P.fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* were used: sterilized seeds (ca 30 seeds); germinated seed (ca 200 mg which varied in size from 3 to 5 mm from shoot tip to root tip); seedling shoots (ca 200 mg seedling shoots with hypocotyls about 5 mm long); and leaves (ca 200 mg from *in vitro* grown plantlets). Explants were cultured in 100 ml Erlenmeyer flasks containing 20 ml MS liquid medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin (abbreviation DK medium).

In another experiment seedling shoots of *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* (ca 200 mg) were used. They were cultured in 100 ml flasks containing 20 ml of MS liquid medium supplemented with combinations of 2,4-D (1.0, 2.0 mg l^{-1}) and BA (0.5, 1.0, 2.5 mg l^{-1}).

The cultures were placed on a gyratory shaker at 100 rpm in a growth room at 25°C in darkness for one week for seedling shoot explants and two weeks for the other explants. Treatments were repeated five times. Each week cell numbers per ml medium and the percentage of viable cells were determined after staining the cells with FDA (Widholm, 1972) using a haematocytometer (0.2 x 1 x 1 mm^3) and 100 x magnification reverse microscope.

2.2.3 Cell suspensions

Aliquots (20 ml) of cell suspensions derived from seedling shoot explants of all 4 species in the two experiments of section 2.2.2 were filtered through a 1000 μm steel mesh and dispensed into 250 ml flasks containing 100 ml of culture medium. For cell suspensions from DK medium the culture media consisted of: (1) DK medium, (2) MS liquid medium supplemented with 2.5 mg l^{-1} NAA and 2.5 mg l^{-1} BA (NB medium), (3) mixtures of DK and NB medium in the following ratios: 1:1, 1:2, 2:1 (v/v, DK_1NB_1, DK_1NB_2, DK_2NB_1). Cell suspensions cultured in MS medium with combinations of 2,4-D and BA were subcultured on fresh medium of the same composition. Treatments were replicated 3 times. Cultures were kept in the same conditions as described in section 2.2.2. For the first two subcultures, 30 ml supernatant containing small cells (about 4 x 10^5 cells/ml) was taken up in a Pasteur capillary pipette after the suspension had settled for 1 min and then dispensed into 250 ml flasks containing 100 ml fresh media. In subsequent subcultures 25 ml of cell suspension cultures were transferred to a new flask containing 100 ml fresh medium. Subcultures were taken when the cell growth cycle was in the stationary phase. The 6th subculture of *P.tomentosa* in DK medium and the 6th and 20th subcultures of *P.taiwaniana* in DK_1NB_2 medium were used to determine the cell growth cycle in terms of pH, fresh and dry weight, settled cell volume (SCV), packed cell volume

(PCV) and optical density (OD) (Davis, *et al.*, 1984; Ryu & Lee, 1990). Samples of approximately 5 ml were withdrawn from each of the 3 replications at one or two day intervals using sterile Pasteur pipettes. pH was determined from 5 ml samples. Settled cell volume was determined after allowing the 5 ml samples to settle for 30 min in graduated conical centrifuge tubes. Packed cell volumes were determined after centrifuging the above samples at 100 xg for 5 min. The absorbances (optical density) at 525 nm of 1 ml samples diluted with fresh culture medium to a final volume of 5 ml were determined with a spectrophotometer (Spectronic 20, Bausch & Lomb.). Fresh weight was determined by filtering the sample through a known preweighed wet millipore filter (45 μm, Millipore Corp., Bedford, MA01730) and washing the filtrate with three volumes of distilled water under vacuum. Dry weight was determined by subtracting the preweight of dry filters from that of cells plus filters after drying at 60^{o}C in an oven for 24 hours.

2.3 Cell and cell aggregate cultures

Cell suspension cultures derived from the early linear growth phase of the 3rd, 6th and 12th subcultures of *P.tomentosa* in DK medium and *P.taiwaniana* in DK_1NB_2 medium were transferred in 10 ml aliquots to centrifuge tubes. Cell densities were adjusted to 10, 5 and 2 x 10^5 cells per ml by dilution with fresh medium. Suspensions were mixed gently with an equal volume of melted agar medium (1.2 % Difco agar) which had been kept at 45^{o}C, and 4 ml or 1 ml aliquots were poured into a 9- or 3-cm Petri dish respectively. The cell densities in Petri dishes were thus 5, 2.5 and 1 x 10^5 per ml medium solidified with 0.6 % agar. Media used were DK medium for *P.tomentosa* and DK_1NB_2 medium for *P.taiwaniana*.

Calli which developed from single cells of both species were subcultured on the same media solidified with 0.6 % agar. Callus pieces were also placed on a microporous polypropylene membrane of a Magenta vessel (Sigma M3029) with 25 ml liquid media. Some of the calli were left in the same Petri dishes but 4 ml fresh liquid media were added and placed on a reciprocal shaker at 60 rpm. Calli derived from *P.taiwaniana* cells were also transferred to 250 ml flasks containing 100 ml DK_1NB_2 medium and resuspended on a gyratory shaker at 100 rpm to select for possible browning-resistant callus lines.

Cell aggregates obtained when suspension cells (25 ml) of the 6th subculture of *P.tomentosa* in DK medium were inoculated into NB medium (100 ml) or *P.taiwaniana* in DK_1NB_2 medium (25 ml) were inoculated into MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA or 1 mg l^{-1} NAA and 10 mg l^{-1} BA (100 ml). Cell aggregates (ca 1-2 mm in diameter) of both species were retrieved after the cultures were sieved through a 1000 μm mesh. They were thin plated into 9 cm Petri dishes containing 4 ml media. The culture media used were MS medium containing 0.5, 1 mg l^{-1} NAA and 5, 10 mg l^{-1} BA solidified with 0.6 % agar. To induce organogenesis callus (ca 10 clumps) derived from the cell aggregates of *P.taiwaniana* were transferred into 9 cm Petri dishes containing 5 ml MS medium containing combinations of NAA (0, 0.1 mg l^{-1}), BA (0, 5, 10 mg l^{-1}) and GA_3 (0, 0.5 1 mg l^{-1}). Callus (ca 5 mm in diameter) were also nurse cultured in test tubes. The nurse tissues consisted of either shoot tip or internodal explants of *P.taiwaniana* cultured on 5 ml MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA solidified with 0.8 % Difco agar. A filter paper disc (6 cm in diameter) was placed on the top of nurse tissue and the callus was placed on the filter paper disc.

RESULTS AND DISCUSSION

3.1 Cell suspension from callus

Compact callus derived from explants of four *Paulownia* species grown in MS medium containing NAA and BA failed to go into suspension. Few cells were released and the cultures soon turned brown. Friable callus obtained from explants of all 4 *Paulownia* species grown in MS medium containing 2,4-D and kinetin also failed to develop into cell suspension cultures in MS liquid medium with 2,4-D (Table 7.1). Cell divisions were very slow and many cells were dead as revealed when stained with FDA. Friable callus of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* cultured in combinations of NAA (2.5, 5.0 mg l^{-1}) and BA (2.5 mg l^{-1}) turned brown after the third subculture. Cell suspension of *P.tomentosa* was established from friable callus cultured in MS medium containing 2.5 mg l^{-1} NAA and 2.5 mg l^{-1} BA. Friable callus was derived from shoot tip explants in MS medium containing 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. Suspensions have been maintained for more than 1.5 years by regular subculturing. Cells were small and spherical (ca 25 μm in diameter) but grew very slowly (Fig. 7.1). The period between subcultures was two months. The very slow increase in fresh and dry weight of the cells prevented quantification of a cell growth cycle.

Table 7.1 Responses of friable callus of *Paulownia* species in liquid culture

Liquid media 2,4-D mg l^{-1}	Origin of callus		suspension cells	Color of media
	Media	Explants		
1.0	$D_{0.1}K_{0.1}$	shoot tip	++	light yellow
0.5			+	light yellow
1.0	$D_{0.01}K_1$	shoot tip	-	-
0.5			-	-
1.0		cotyledon	+,rooting	light yellow
0.5			-,rooting	-
1.0		hypocotyl	+++	light yellow
0.5			++	light yellow
1.0		root		-
0.5			rooting	-
1.0	$D_{0.1}K_1$	hypocotyl	++	light yellow
0.5			+,rooting	light yellow

-, +, ++, +++ represent non, few, medium, large numbers of cells released from friable callus
$D_{0.1}K_{0.1}$: 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin
$D_{0.01}K_1$: 0.01 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin
$D_{0.1}K_1$: 0.1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin

Fig. 7.1. Cell suspension derived from friable callus of shoot tips of *P.tomentosa*. Cell suspension maintained for 1.5 years by regular subculture in NB medium. Bar represents 50 μm.

3.2 Cell release from different explants

Cells released readily from the developing callus tissues on all the explants in DK medium and seedling shoot explants in media containing combinations of 2,4-D and BA. Cells of *P.fortunei*, *P.kawakamii* and *P.taiwaniana* explants differed considerably in size and shape. They ranged from small to large in size and round to long or spiral in shape (Fig. 7.2-a). The media took on a deep yellow color in the case of seed and seedling explants and a light green color for shoots and leaf explants. Cells of *P.tomentosa* were more homogeneous in shape (small and elongated, Fig. 7.2-b and c) and the medium became light green in color regardless of explant type. The number of cells released per ml per 200 mg of explants in DK medium were about the same (4-5 x 10^5 cells) for the four species (Fig. 7.3-a). Most of the cells released were alive except for *P.fortunei* where only 20 % of the cells were alive (Fig. 7.3-b). Cells from young and non-lignified tissues of seedling shoots went into suspension after one week of culture in DK medium. Cells from leaf explants required a further week.

The number of cells released from seedling shoot explants and their viability in media containing 2,4-D and BA varied and were species dependent (Fig. 7.4). For *P.kawakamii* and *P.taiwaniana* the number of cells released and viability in 2,4-D and BA media equalled that of DK medium. In the case of *P.tomentosa* cell numbers of 8-10 x 10^6 cells/ml and a viability percentage of >80 were achieved in the combination of 2,4-D (1.0 and 2.0 mg l^{-1}) and BA (0.5 mg l^{-1}).

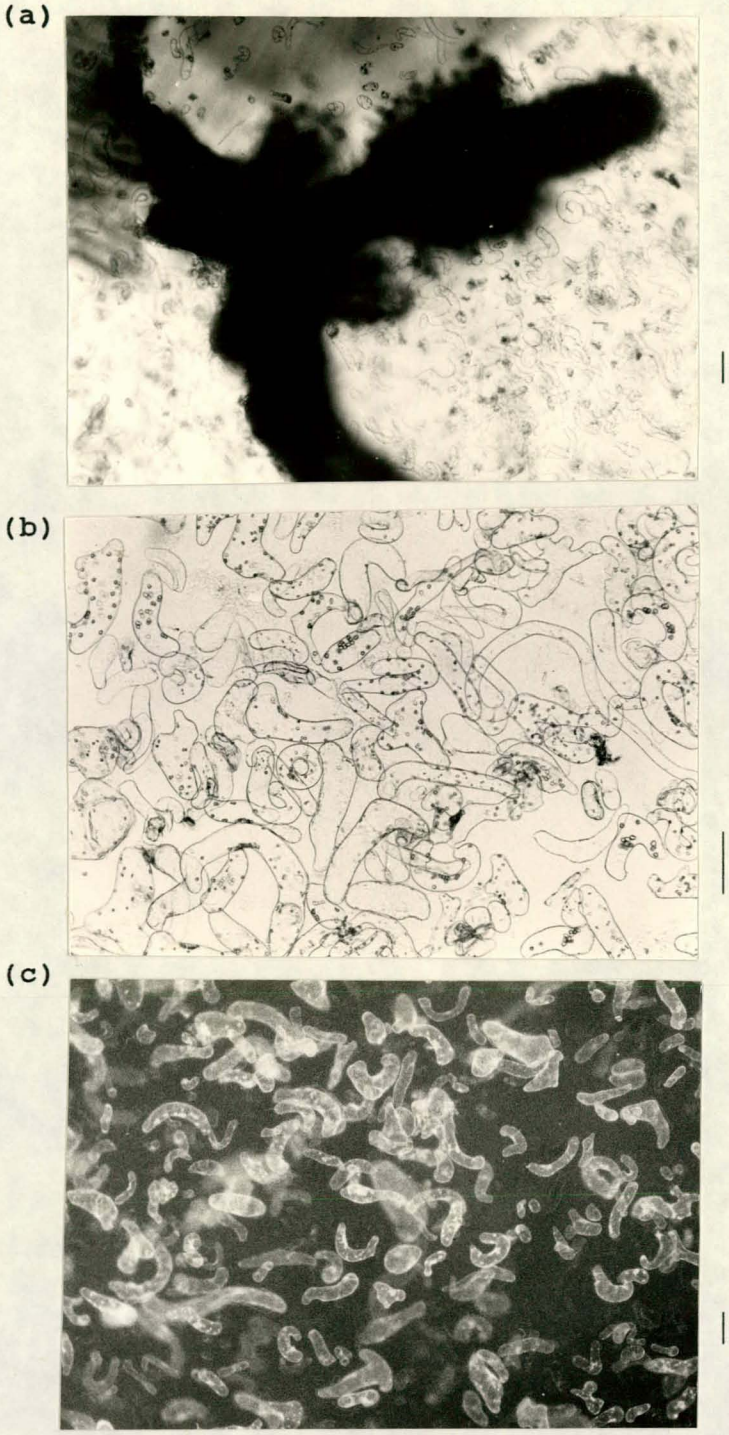


Fig. 7.2. Cell release and viability on MS medium containing 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin: (a) cells released from germinating embryo of seed culture of *P.taiwaniana*; (b) Cells released from seedling shoots of *P.tomentosa*, mesophyll cell, chloroplasts are evident; (c) cells stained with FDA. Cell viabilities exceeded 90 %. Bar represent 50 μm.

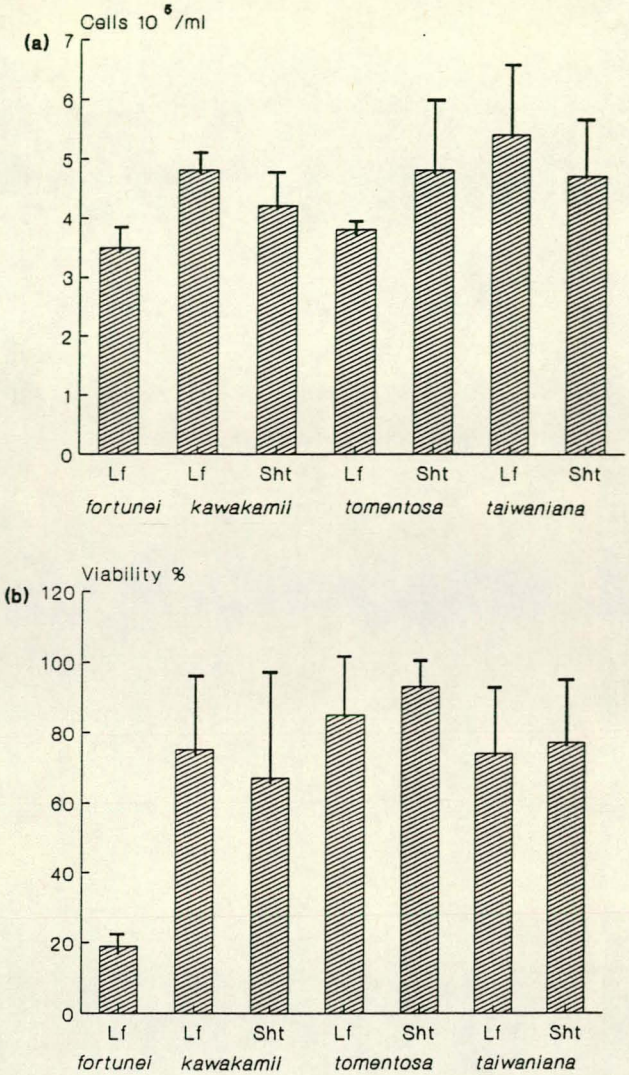


Fig. 7.3. Cells released from *Paulownia* explants on MS medium containing 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin: (a) cell densities, (b) cell viability. Lf: plantlet leaves, Sht: seedling shoots

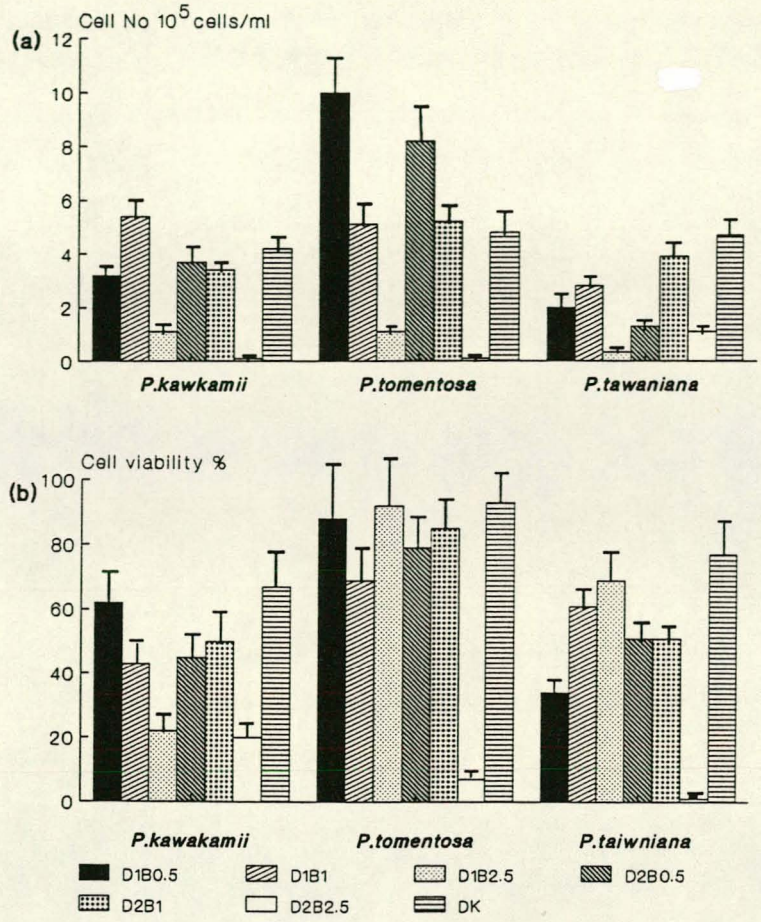


Fig. 7.4. Cells released (a) densities and (b) viability % from seedling shoots in 2,4-D and BA combination for three *Paulownia* species. D1B0.5: 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA, D1B1: 1 mg l^{-1} 2,4-D and 1 mg l^{-1} BA, D1B2.5: 1 mg l^{-1} 2,4-D and 2.5 mg l^{-1} BA, D2B0.5: 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA, D2B1: 2 mg l^{-1} 2,4-D and 1 mg l^{-1} BA, D2B2.5: 2 mg l^{-1} 2,4-D and 2.5 mg l^{-1} BA, DK: 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin

3.3 Cell suspensions

Cells of *P.fortunei* and *P.kawakamii* gradually lost their viability (Fig. 7.5-a) and died before the third subculture irrespective of explant types and culture media. Cells of *P.taiwaniana* derived from seeds, germinating seeds and leaves also died before the third subculture in all media tested. However, cells from seedling shoots in DK_1NB_2 medium were maintained by serial subculturing for 1.5 years. Cells of *P.tomentosa* derived from all explants were successfully maintained by regular subculturing in DK medium.

The cell growth cycle of *P.tomentosa* for the sixth subculture revealed an initial slow but linear growth phase for 4 days (Fig. 7.6-a). This was followed by a rapid and linear growth phase which lasted for 10 days, a stationary phase then set in. Dry weight doubled in 8 days during the rapid linear growth phase. Cell growth in terms of fresh and dry weight correlated with optical density (OD) or cell settled volume (CSV) during the linear growth phase. Optical density is a rapid and accurate method for determining cell mass of cell suspension cultures (Eriksson, 1965; Miller, *et al.*, 1968; Kurz, 1971; Kubek & Shuler, 1978). However, the colour of the suspension cultures turned dark brown during late linear phase and the high OD values obtained no longer gave an accurate simulation of the increase in fresh and dry weight. CSV is also used as a method to determine cell growth, it is, however, time consuming and not sensitive enough to identify the beginning of the stationary phase.

Cells of *P.tomentosa* were generally spherical in shape and varied from 50-75 μm in diameter. During cell division cells formed one or more protuberances which developed into new cells (Fig. 7.5-b) or they divided along the equatorial axis to form 2 daughter cells.

Suspension cultures of *P.taiwaniana* were maintained for more than 1.5 years by regular subculture in DK_1NB_2 medium. The cell cycle of the sixth subculture of *P.taiwaniana* was shorter than that of *P.tomentosa* (Fig. 7.6-b). No obvious lag phase both in fresh and dry weight was observed. The linear growth phase lasted 8 days and the dry weight doubled in 2.5 days. The high cell densities in these cultures resulted in poor correlation between OD and the cell growth in terms of fresh and dry weight. Diluting cultures to the extent of 1:5 ml to 1:10 ml increased the variation of OD readings but did not improve the accuracy. PCV measurement was quicker than SCV. But like SCV, it became less accurate during the latter part of the linear growth phase when fresh and dry weight increased more rapidly than cell volume. The pH of the cultured media declined from 5.5 to 4.7 during the culture period until the beginning of the stationary phase. pH again increased from 4.7 to 5.3 during the latter part of the stationary phase allowing it to be clearly identified this phase.

Two cell types of *P.taiwaniana* dominated the cultures. Elongated cells (150 μm long and 25 μm wide, Fig. 7.5-c) and small spherical cells (25 μm in diameter, Fig. 7.5-d). Towards the end of the linear growth phase, cells increased in size and were generally more than 200 μm in diameter (Fig. 7.5-e). During the linear growth phase, elongated cells formed a row of cells within a mother cell (from 2-10 cells, Fig. 7.5-c) which were released when the cell wall of the mother cell ruptured. This behavior is comparable to that reported for sycamore (*Acer pseudoplatanus*) cell suspensions (Henshaw *et al.*, 1966). Spherical cells divided into 'dense moruloid masses' (Fig. 7.5-d) similar to that described for carrot cultures by Steward *et al.* (1958). These differences in behavior were related to auxin and cytokinin content of the medium. Single elongated cells dominated in DK medium whereas spherical cell clumps dominated in NB medium. Combination of DK and NB medium in the ratio of 1:2 (v/v) resulted in more free cells and fewer cell clumps of *P.taiwaniana*. After 12

subcultures spherical cells were gradually replaced by elongated cells and the growth cycle also changed. The pattern of cell growth cycle of the 20th subculture was similar to the 6th subculture (Fig. 7.6-c) but the time required for the cultures to double the fresh and dry weight was longer. A clear exponential, a linear and a short stationary phase was evident in fresh weight of culture, whereas dry weight and PCV revealed only the latter two phases. Dry weight and PCV doubled in 8 days and the cycle lasted 15 days. The pH of the medium (ca 4.5 - 4.7) was stable and constant throughout the growth cycle.

Cell suspension cultures of *P.tomentosa* and *P.taiwaniana* were established within one month by culturing explants directly. This eliminates the production of a friable callus interphase on agar media which turned brown when subcultured.

The high growth rates and length of the growth cycle for cell suspension cultures of *P.tomentosa* and *P.taiwaniana* are comparable to other woody plants such as *Acer pseudoplatanus* (Givan & Collin, 1967), *Eucalyptus gunnii* (Teulieres *et al.*, 1989), and *Populus alba* (Park & Son, 1988).

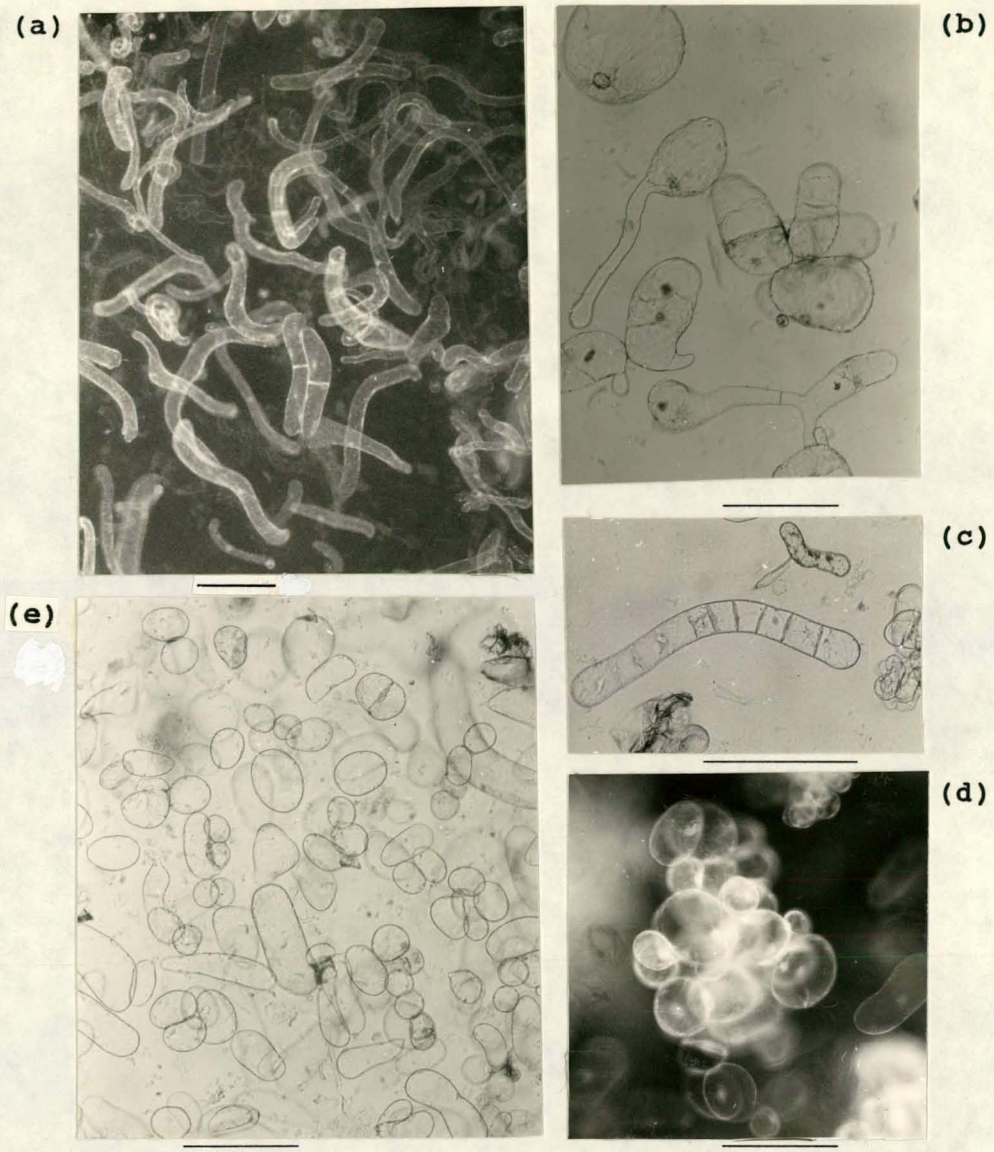


Fig. 7.5. Suspension cells: (a) elongated cells from leaves of *P.fortunei* stained with FDA, viability ca 70 % at the 2nd subculture on DK medium; (b) large spherical cells of *P.tomentosa* of the 6th subculture on DK medium, new cells form from protuberances or dividing along the equatorial axis of cell; (c-d) *P.taiwaniana* cells: (c) elongated cell divide to formed a row of cells and (d) moruloid cell mass (staining with FDA) and (e) cells during the stationary phase at the 6th subculture. Bars represent 50 μm.

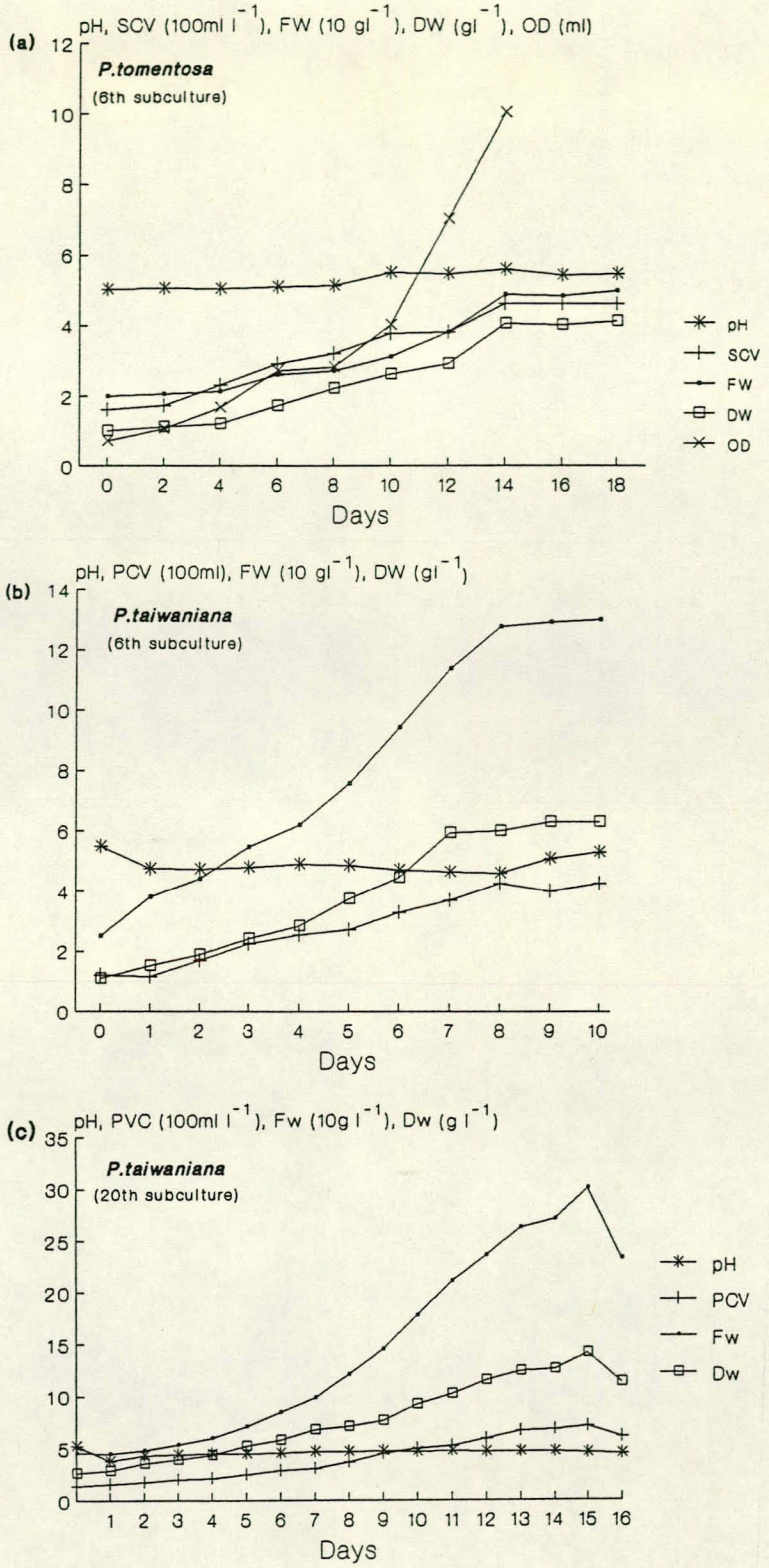


Fig. 7.6. Cell growth cycle of *P.tomentosa* for the 6th subculture (a) and *P.taiwaniana* for the 6th (b) and 12th (c) subculture. SCV: settled cell volume, PCV: packed cell volume, FW: fresh weight, DW: dry weight, OD: optical density at 525 nm

3.4 Cell and cell aggregate culture

Cells from the 3 rd subculture of *P.tomentosa* plated on DK medium formed callus (Fig. 7.7). Cytoplasm became dense and the nuclear chromosomes were clearly visible in the dividing cells (Fig. 7.7-b and c). Only one callus colony formed when cells of a density of 2.5 x 10^5 cells/ml where planted out, at the other two densities of cultures, cells divided but did not form callus. Callus subcultured on DK medium solidified with agar or on microporous polypropylene membrane rafts suspended above the liquid DK medium turned brown and died. Calli also turned brown when fresh liquid medium was added to the cultures left in Petri dishes. Cells of the 6th subculture of *P.tomentosa* suspension cultures consisted mainly of single giant spherical cells. They failed to form callus. Cells with a density of 5 x 10^5 cells/ml of *P.taiwaniana* plated in DK_1NB_2 medium formed many cell colonies. Fewer colonies formed when lower cell densities were used. Like callus subculture of *P.tomentosa*, cell colonies of *P.taiwaniana* failed to grow and turned brown when subcultured on DK_1NB_2 medium with agar or on polypropylene membrane rafts suspended on liquid DK_1NB_2 medium or when fresh liquid medium was added to callus cultures maintained in Petri dishes. Cell colonies resuspended in DK_1NB_2 liquid medium to select possible browning-resistent cell lines also failed. The cultures turned brown after the 3rd subculture.

When cells of the 6th subculture of *P.tomentosa* on DK medium were transferred to NB medium, cell division was induced. One cell could form up to 50 daughter cells (Fig. 7.8-a). The cell growth cycle increased rapidly from the 6th day when many cell aggregates formed (Fig. 7.9). Subculture of cell aggregates in MS medium containing combinations of NAA and BA however failed to grow further, turned brown and died. Many cell aggregates formed when suspension cells of *P.taiwaniana* in DK_1NB_2 medium were transferred into MS medium containing combinations of NAA and BA.

The ability to form cell colonies from single cells and cell aggregate formation in liquid medium containing NAA and BA were lost by the 12th subculture. Subculture of cell aggregates in MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA or 1 mg l^{-1} NAA and 10 mg l^{-1} BA formed callus (Fig. 7.7-b) and occasionally roots differentiated. They failed to form shoots when subcultured in media containing GA_3 or using nurse culture (Fig. 7.8-c).

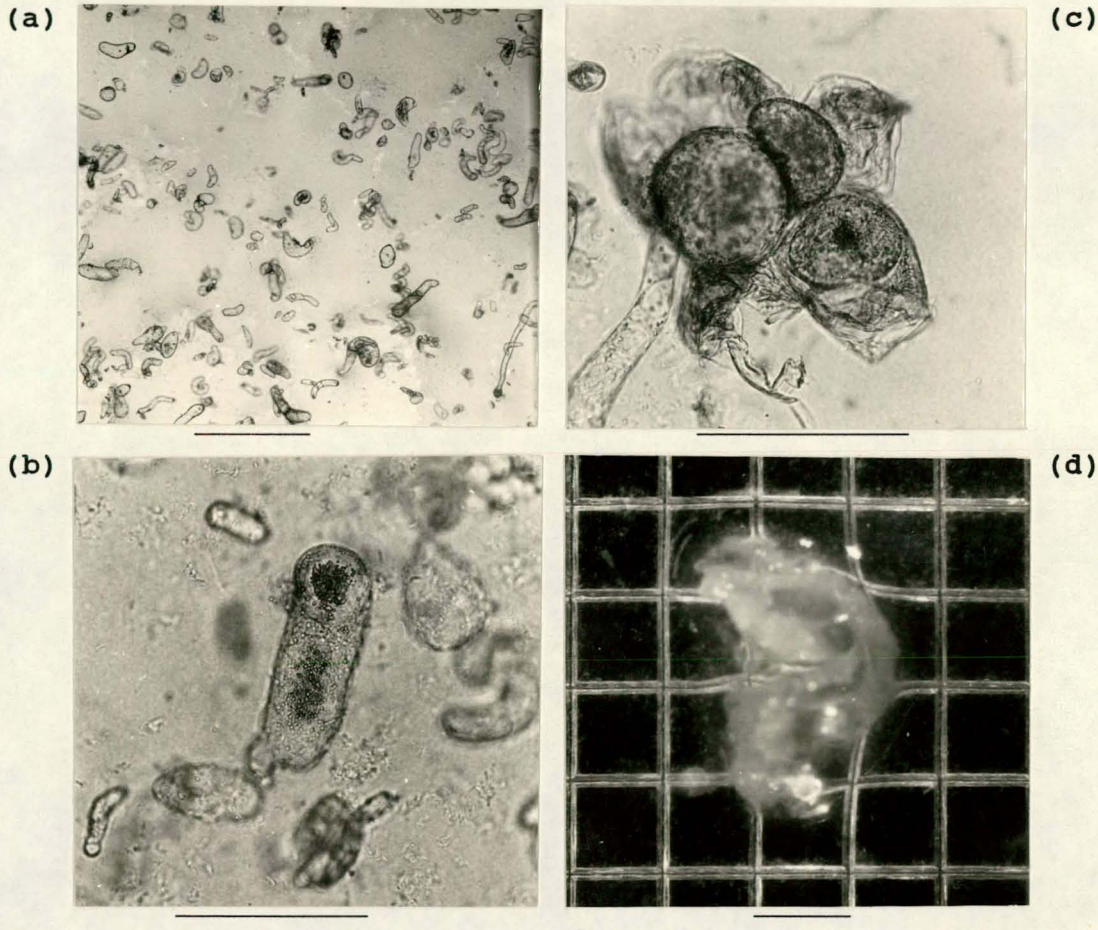


Fig. 7.7. Cell culture of P.*tomentosa*: (a) suspension cells of 1.0 x 10^5 per ml plated on agar in Petri dish, (b) dividing cell, (c) small cell clumps, and (d) callus formation. Bars represent 100 μm, except the bar of (d) represents 2 mm.

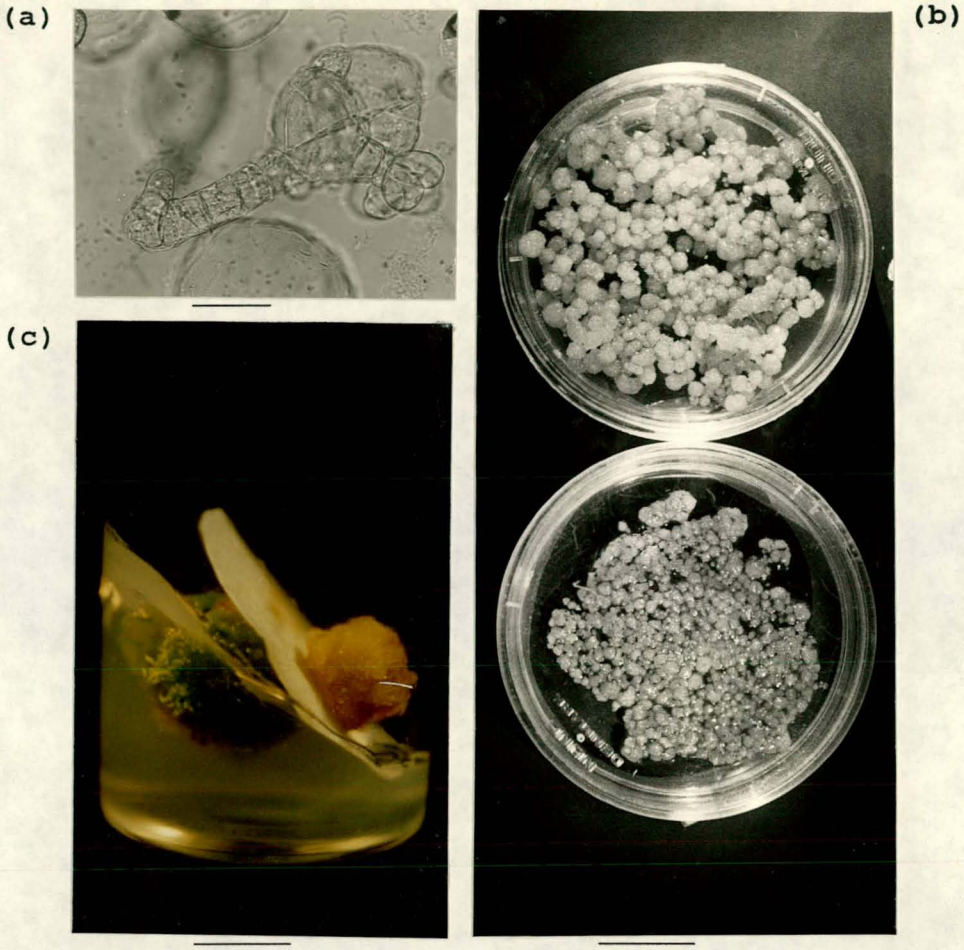


Fig. 7.8. Cell aggregates: (a) suspension cells of *P.tomentosa* in DK medium transferred to NB medium induced cell division up to 50 cells formed per mother cell, (b) suspension cells of *P.taiwaniana* in DK_1NB_2 medium transferred to NB medium formed globular cell clumps which were then plated on agar in Petri dishes, (c) callus of *P.taiwaniana* nurse cultured on shoot tip explants of *P.taiwaniana*. No organogenesis occurred in the callus. Note proliferous shoot formation of nurse explant. The bar in (a) represents 25 μm, the other bars represent 2 cm.

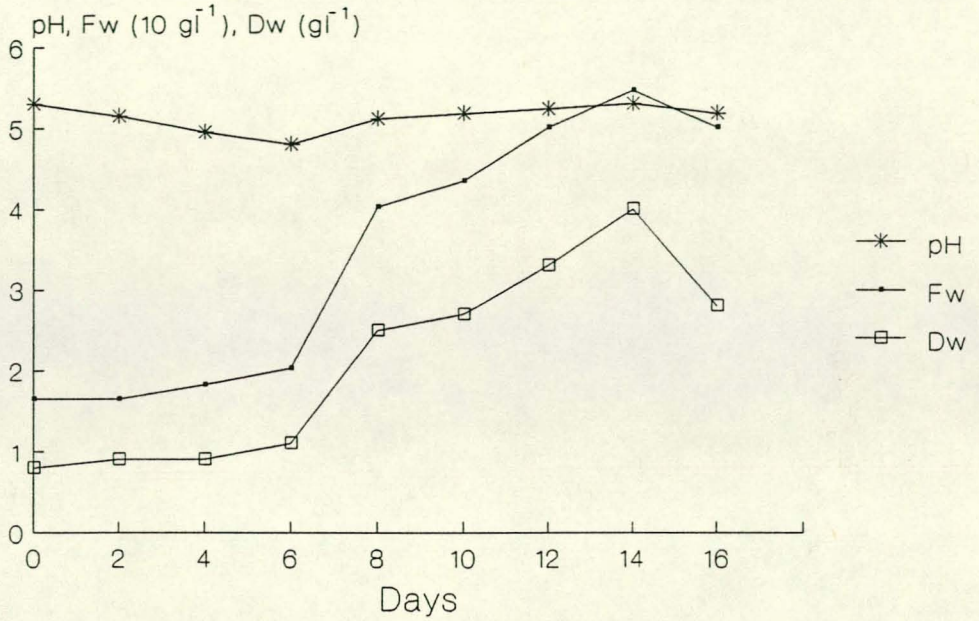


Fig. 7.9. Cell growth cycle of *P.tomentosa* when suspension cells in DK medium were transferred to NB medium. Fw: fresh weight, Dw: dry weight

4. REFERENCES

Attree, S.M., Bekkaoui, F., Dunstan, D.L. and Fowke, L.C., 1987. Regeneration of somatic embryos from protoplasts isolated from an embryogenic suspension culture of white spruce (*Picea glauca*). Plant Cell Rep. 480-483

Bekkaoui, F., Saxena, P.K., Attree, S.M., Fowke, L.C. and Dunstan, D., 1987. The isolation and culture of protoplasts from an embryogenic cell suspension culture of *Picea glauca* (Moench) Voss. Plant Cell Rep. 6:476-479

Bernard, T., Thoiron, A., Guiel, J.L., Luttge, U. and Thellier, M., 1979. Solute uptake of *Acer pseudoplatanus* cell suspensions during recovery from gas shock. Physiol. Plant. 46:352-356

Brown, E.G. and Short, K.C., 1969. The changing nucleotide pattern of sycamore cells during culture in suspension. Phytochem. 8:1365-1372

Carrier, D-J., Chauret, N., Mancin, M., Coulunbe, P., Neufeld, R., Weber, M. and Arhambault, J., 1991. Detection of ginkgolide A in *Ginkgo biloba* cell cultures. Plant Cell Rep. 10:256-259

Carrier, D-J., Cosentino, G., Neufeld, R., Rho, D., Weber, M. and Archambault, J., 1990. Nutritional and hormonal requirements of *Ginkgo biloba* embryo-derived callus and suspension cell culture. Plant Cell Rep. 8:635-638

Cheema, G.S., 1989. Somatic embryogenesis and plant regenerating from cell suspension and tissue cultures of mature himalayan poplar (*Populus ciliata*). Plant Cell Rep. 8:124-127

Davis, D.G., Stolzenberg, R.L. and Dusky, J.A., 1984. A comparison of various growth parameters of cell suspension cultures to determine phytotoxicity of Xenobiotics. Weed Sci. 32:235-242

Eriksson, T., 1965. Study on the growth requirements and growth measurements of cell cultures of *Haplopappus gracilis*. Physiol. Plant. 18:976-993

Finer, J.J., Kriebel, H.B. and Becwar, M.R., 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). Plant Cell Rep. 8:203-206

Givan, C.V. and Collin, H.A., 1967. Studies on the growth in culture of plant cells II. Changes in respiration rate and nitrogen content associated with the growth of *Acer pseudoplatanus* L. cells in suspension culture. J. Exp. Bot. 18(55):321-331

Hellergren, J., 1983. Cold acclimation of suspension cultures of *Pinus sylvestris* in response to light and temperature treatments. Plant Physiol. 72:992-995

Henshaw, G.G., Jha, K.K., Mehta, A.R., Shakeshaft, D.J. and Street, H.E., 1966. Studies on the growth in culture of plant cells: 1. Growth patterns in batch propagated suspension cultures. J. Exp. Bot. 17:362-377.

Huang, L.C., Huan, M.L. and Chen, W.L., 1990. Tissue culture investigation of bamboo V: Recovery of callus from protoplasts of suspension-cultured *Bambusa* cells. Bot. Bull., Academ. in Sinica 31:29-43

Hurwitz, C.D. and Agrios, G.N., 1984. Isolation and culture of protoplasts from apple callus and cell suspension cultures. J. Am. Soc. Hort. Sci. 109:384-350

Hustache, G., Barnoud, F. and Joseleau, J-P., 1986. Callus formation and induction of a cell suspension culture from *Acacia senegal*. Plant Cell Rep. 5:365-367

King, P.J., 1980. Cell proliferation and growth in suspension cultures. Int. Rev. Cytol. Suppl. 11A:25-53

King, P.J., Cox, B.J., Fowler, M.W. and Street, H.E., 1974. Metabolic events in synchronized cell cultures of *Acer pseudoplatanus* L. Planta 117:109-112

King, P.J., Mansfield, K.J. and Street, H.E., 1973. Control of growth and cell division in plant cell suspension cultures. Can. J. Bot. 51:1807-1823

Kubek, D.J. and Shuler, M.L., 1978. A rapid quantitative method to measure growth of plant cell suspension cultures. Can. J. Bot. 56:2340-2343

Kumar, A., Tandon, P. and Sharma, A., 1991. Morphogenetic response of cultured cells of cambial origin of a mature tree - *Dalbergia sissoo* Rorb. Plant Cell Rep. 9:703-706

Kurz, W.G.W., 1971. A chemostat for growing higher plant cells in single cell suspension cultures. Exp. Cell Res. 64:476-479

Matsuta, N.T., Hirabayashi, T., Akihama, T. and Kozaki, I., 1986. Callus formation from protoplasts of peach cell suspension cultures. Sci. Hort. 28:59-64

Messner, B., Boll, M. and Berndt, J., 1991. L-phenylalanine ammonia-lyase in suspension culture cells of spruce (*Picea abies*). Plant Cell, Tissue & Organ Cult. 27:267-274

Miller, R.A., Wilkinson, A. and Kirkpatrick, J.W., 1968. The measurement of cell density in suspension cultures using dichromate oxidation. Can. J. Bot. 46:1083-1085

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497

Orihara, Y. and Furuya, T., 1990. Production of theanine and other γ-glutamyl derivatives by *Camellia sinensis* cultured cells. Plant Cell Rep. 9:65-68

Park, Y.G. and Son, S.H., 1988. Regeneration of plantlets from cell suspension culture derived callus of white poplar (*Populus alba* L.). Plant Cell Rep. 7:567-570

Ryu, D.D.Y. and Lee, S.O., 1990. Determination of growth rate for plant cell cultures: comparative studies. Tech. & Bio. 35:305-311

Steward, F.C., Mapes, M.O. and Smith, J., 1958. Growth and organized development of cultured cells: 1. Growth and division of freely suspended cells. Am. J. Bot. 45:693-703

Tate, J.L. and Payne, G., 1991. Plant cell growth under different levels of oxygen and carbon dioxide. Plant Cell Rep. 10:22-25

Teulières, C., Feuillet, C. and Boudet, A.M., 1989. Differential characteristics of cell suspension cultures initiated from *Eucalyptus gunnii* clones differing by their frost tolerance. Plant Cell Rep. 8:407-410

Touchet, B., Dukval, Y. and Pannetier, C., 1991. Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq.). Plant Cell Rep. 10:529-532

Tremblay, F.M., 1988. Callus formation from protoplasts of *Betula papyrifera* Mauch. cell suspension culture. J. Plant. Physiol. 133:247-251

Tremblay, F.M., Power, J.B. and Lalonde, M., 1985. Callus regeneration from *Alnus incana* protoplasts isolated from cell suspensions. Plant Sci. 41:211-216

Widholm, J., 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47:189-194

Wilson, H.M. and Street, H.E., 1975. The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis*. Ann. Bot. 39:671-682

Winton, L.L., 1968. The rooting of liquid-grown aspen callus. Am. J. Bot. 55:159-167

CHAPTER 8

PROTOPLAST ISOLATION AND CULTURE

Key words: cell suspension, *Paulownia* species, protoplast isolation, protoplast culture

ABSTRACT

Isolation of protoplasts from *in vitro* explants of *P.tomentosa*, *P.kawakamii* and *P.taiwaniana* and suspension cells of *P.taiwaniana* were studied. Leaves of *P.tomentosa* treated with Seravac pectinase yielded more free mesophyll cells than other brands of pectinase. High yields of protoplasts were obtained when explants were incubated in an enzyme solution consisting of 0.6 % pectinase, 0.6 % hemicellulase and 2 % Onozuka R10 cellulase. High yields of protoplasts from suspension cells of *P.taiwaniana* were obtained when cells were treated with 2 % Seravac cellulase. Suspension cells of *P.taiwaniana* exhibited strong membrane-to-cell wall attachment and many cells failed to plasmolyze completely in a mannitol solution. Injury to the cells was evident in concentrations in excess of 0.6 M mannitol and incubation times longer than 5 hours. Protoplasts from mesophyll cells of *P.tomentosa* failed to divide while initial stages of division in protoplasts from suspension cells of *P.taiwaniana* were evident but division was not completed.

ABBREVIATIONS USED

BA = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; FDA = fluorescein diacetat; MES = 2-N-morpholino ethane sulfonic acid; NAA = α-naphthaleneacetic acid; PVP = polyvinylpyrrolidone

1. INTRODUCTION

Protoplast culture offers novel ways of plant improvement through somatic hybridization by cell and nuclear fusion between two genotypes, or through genetic engineering by the incorporation of specific portions of foreign genetic material into a target genome. According to Dunstan and Thorpe (1986), there were only two reports of the successful regeneration of plants from protoplasts of woody tree species: *Citrus* (Vardi *et al.*, 1982), and *Santalum album* (Rao & Ozias-Akins, 1985). Since then successful regeneration of plants from protoplasts have been reported on *Broussonetia kazinoki* (Oka & Ohyama, 1985), *Pyrus communis* var. *pyraster* (Ochatt & Caso, 1986), Ulmus x 'Pioneer' (Sticklen *et al.*, 1986), *Coffea canephora* (Schöpke *et al.*, 1987), *Malus* x *domestica* (Patat-Ochatt *et al.*, 1988), *Solonum dulcamara* (Chand *et al.*, 1988), *Prunus* species (Ochatt *et al.*, 1987, Ochatt & Power, 1988, Durzan, 1988; Ochatt, 1990), *Actinidia chinensis* var. *chinensis* (Tsai, 1988), Shrubby *Oxalis* species (Ochatt *et al.*, 1989), *Duboisia myoporoides* (Kitamura *et al.*, 1989), *Populus alba* x *P.grandidentata* 'Crandon', NC-5339, *P.nigra* 'Betulifolia' x *P.trichocarpa*, NC-5331, and *P.tremula* 'Erecta' (Russell & McCown, 1988; Park & Son, 1992), *Pinus taeda* (Gupta & Durzan, 1987), and *Picea glauca* (Attree *et al.*, 1987). High yields of protoplasts isolated from 1 year old seedlings of *P.taiwaniana* and *P.fortunei* grown in a greenhouse have been reported (Yang *et al.*, 1989; Saito 1980 a and b). The viability of protoplasts is affected by the growth conditions of the donor plants such as season,

light intensity, nutritional states, age, etc. Therefore *in vitro* grown shoots have been used extensively as a source for the isolation of protoplasts (Evans & Bravo, 1983). The isolation and culture of protoplasts from *in vitro* grown shoot explants and cell suspension of *Paulownia* are reported here.

2. MATERIAL AND METHODS

2.1 Protoplasts isolated and cultured from *in vitro* cultures

2.1.1 Effect of pectinase brand and concentration on cell isolation

Leaves of *in vitro* plantlets of *P.tomentosa* derived from successive shoot tip subcultures were used. Leaves (200 mg) were cut into 1-2 mm strips and incubated in 6-cm Petri dishes containing 5 ml MS medium supplemented with each of the pectinase brands (A to D) and addenda (G) as shown in Table 8.1. Petri dishes incubated in a water bath (30^{o}C) placed in the dark on a reciprocal shaker at 60 rpm for 5 hours or left in the dark for 15 hours without shaking. The number of mesophyll cells was counted using a hematocytometer.

In this series of trials Petri dishes were sealed with Parafilm when incubated on enzyme solution or kept in culture containers. All treatments were repeated three times.

Table 8.1. Media composition of enzyme solution

Enzyme solution and addenda	Concentration
Basal media	
MS or DK_1NB_2[1] medium	
Enzymes	
(A) Pectinase (Sigma chemical Co.)	0.3, 0.6 or 1.0 %
(B) Pectinase (Seravac, RSA)	0.3, 0.6 or 1.0 %
(C) Macerozyme R10 (Seravac, RSA)	0.3, 0.6 or 1.0 %
(D) Serazyme (Seravac, RSA)	0.3, 0.6 or 1.0 %
(E) Cellulase (Seravac Co. RSA)	1, 2 or 3 %
Hemicellulase (Sigma chemical Co.)	0.6 %
Pectinase (Seravac, RSA)	0.6 %
(F) Cellulase (Seravac or Onozuka R10[2])	2 %
Hemicellulase (Sigma chemical Co.)	0.6 %
Pectinase (Seravac)	0.6 %
Addenda	
(G) Mannitol	0.6 M (54.7 g l^{-1})
PVP	200 mg l^{-1}
$CaCl_2 \cdot H_2O$	4.5 mM (0.5 g l^{-1})
MES (2-N-mopholino ethane)	3 mM (640 mg l^{-1})

pH of enzyme solutions was adjusted to 5.5 before being subjectd to filter sterilization through a 0.45 μm millipore.

DK_1NB_2 medium consisted of MS medium containing 0.3 mg l^{-1} 2,4-D, 0.03 mg l^{-1} netin, 1.6 mg l^{-1} NAA and 1.6 mg l^{-1} BA, [2]Onozuka R10 cellulase was made from akult Honsha Co., Japan

2.1.2 Protoplast isolation

2.1.2.1 Effect of cellulase concentration

Seedling shoots (200 mg) of *P.kawakamii* were cut into 1-2 mm sections and placed into a 6 cm Petri dishes containing 5 ml MS medium supplemented with enzymes (E) and addenda (G) as shown in Table 8.1. A similar amount of shoot tissues was placed in MS medium containing 0.6 M mannitol and 4.5 mM $CaCl_2 \cdot 2H_2O$ for 1 hour before incubation in an enzyme solution. Petri dishes were incubated on a water bath shaker for 5 hours as described in section 2.1.1. Pure and washed protoplast suspensions were obtained as follows: protoplasts and enzyme suspensions were filtered through a 60 μm mesh; the filtrate was then centrifuged at 100 xg for 7 min and the supernantant removed with a Pasteur pipette; the residue of protoplast suspension was transferred gently to a centrifuge tube containing 10 ml of 21 % sucrose and centrifuged at 100 xg for 7 min; the band of floating protoplasts was collected with a Pasteur pipette and transferred into centrifuged tubes; the enzyme solution was removed by resuspending the protoplasts in wash medium containing MS medium with addenda (Table 8.2) and centrifuged for 7 min at 100 xg. This procedure was repeated 3 times. The number of protoplasts per g of explants was counted using a haematocytometer.

2.1.2.2 Effect of cellulase brand

Leaves (200 mg) of *in vitro* grown plantlets of *P.tomentosa* were cut into 2-3 mm strips and placed into 6-cm Petri dishes containing MS medium supplemented with enzymes (F) and addenda (G) as shown in Table 8.1. Petri dishes were incubated on a water bath shaker for 5 hours as described in section 2.1.2.1. or incubated in dark for 15 hours without shaking. Pure and washed protoplast suspensions were obtained in the

same way as described in section 2.1.2.1. The number of protoplasts were counted using a haematocytometer.

Table 8.2. Composition of wash medium

Basal media and addenda	Concentration
Basal media	
MS medium or DK_1NB_2[1] medium	
Addenda	
Mannitol	0.6 M (54.7 gl^{-1})
$CaCl_2 \cdot H_2O$	4.5 mM (0.5 gl^{-1})

pH of wash media was adjusted to 5.7 before autoclaved at 121°C for 15 min.

[1]DK_1NB_2 medium consisted of MS medium containing 0.3 mg l^{-1} 2,4-D, 0.03 mg l^{-1} kinetin, 1.6 mg l^{-1} NAA and 1.6 mg l^{-1} BA.

2.1.3 Protoplast culture

Pure and washed protoplast suspensions were obtained from leaf explants of *P.tomentosa* as described in section 2.1.2.2 , using Onozuka R10 cellulase. Protoplast density was adjusted to 4 x 10^5 per ml by adding fresh medium. Multidishes (Falcon 3070, Becton Dickinson Labware) with 12 x 8 culture wells were used to culture protoplasts by the microdrop array technique (Potrykus *et al.*, 1979). As each well contained 60 μl culture media and 20 μl protoplast suspension, the final protoplast density was 1 x 10^5 per ml. The culture media consisted of MS medium plus 0.6 M mannitol and 4.5 mM $CaCl_2 \cdot 2H_2O$ and combinations of NAA (0.5, 1.0, 2.5 mg l^{-1}) or 2,4-D (0.1, 0.5, 1 mg l^{-1}) and BA (0.5, 2.5, 5 mg l^{-1}). Multidishes were sealed by

Parafilm and then placed in a closed container with wet filter papers on its floor to maintain a high humidity. Protoplast growth was observed under a inverted microscope.

2.2 Protoplast isolation from suspension cells

2.2.1 Effect of mannitol concentration on cell plasmolysis

Cell suspension of *P.taiwaniana* at the 12th subculture (as described in Chapter 7) were subcultured twice with a 7 day interval to stimulate cell division. Cell suspensions were diluted 1:4 (25 ml suspension added to 75 ml fresh medium). Cell suspensions in the exponential growth phase (4-5 day after transfer) were allowed to settle for 15 min. One milliliter of cell suspension cells was transferred to 6 cm Petri dishes containing 5 ml media. Media consisted of MS medium containing 0.3 mg l^{-1} 2,4-D , 0.03 mg l^{-1} kinetin, 1.6 mg l^{-1} NAA and 1.6 mg l^{-1} BA (DK_1NB_2 medium) plus 0, 0.5, 0.6, 0.7, 0.8 M mannitol (molecular weight 182.17). Petri dishes were placed in a reciprocal shaker (60 rpm, 25^{o}C) in darkness for 15 hours. Cell viability was determined after 1, 3, 5, and 15 hours in culture by staining the cells with FDA. Dead and live cells were counted in randomly selected microscope fields (ca 100 cells were counted) with a microscope with 40 x magnification.

2.2.2 Protoplast isolation

Aliquots (1 ml) of cell suspension of *P.taiwaniana* (as described in 2.2.1) were transferred to 6-cm Petri dishes containing 5 ml DK_1NB_2 medium supplemented with enzymes (F) and addenda (G) as shown in Table 8.1. Petri dishes were incubated for 5 hours on a water shaker bath as described in section 2.1.1. Pure and washed

protoplasts were obtained in the same way as described in section 2.1.2.1. Wash medium used was DK_1NB_2 medium. The number of protoplasts were counted as previously described.

2.2.3 Protoplast culture

Pure and washed protoplast suspensions of *P.taiwaniana* were obtained as described in section 2.2.2. Onozuka R10 cellulase was used. Protoplast density was 1 x 10^5 per ml. Ten drops of protoplast suspension were placed singly in the lid of a 9-cm Petri dish using a Pasteur pipette (hanging drop culture). Petri dishes were placed in a closed container and kept in the same culture condition as described in section 2.1.3. Protoplast division was observed under a inverted microscope.

3. RESULTS AND DISCUSSION

3.1 Effect of pectinases on mesophyll cell isolation

Incubation of leaf strips of *P.tomentosa* for 5 hours on a shaker in Seravac pectinase caused many mesophyll cell clumps to be released (Table 8.3). Similar treatment with other brands of pectinase failed to cause the development of cell clumps. A yield of 10 x 10^5 cells or more was obtained when Seravac pectinase was used at a concentration of 0.6% or higher after stationary incubation for 15 hours.

Table 8.3. Effect of concentration and incubation time of different pectinases on the release of mesophyll cells of *P.tomentosa* leaves.

Pectinases	Incubation time (hours)	Number of cells x 10^5 released from 1 g leaves		
		pectinase concentration		
		0.3 %	0.6 %	1.0 %
Sigma	5[1]	-	-	+
	15[2]	1.7	6.0	6.0
Seravac	5	++	+++	+++
	15	5.5	10.0	12.5
Macerozyme	5	-	-	+
	15	5.0	6.0	7.5
Serazyme	5	-	-	-
	15	0.5	0.5	5.0

-, +, +++ represent none, few, many pieces of mesophyll released from leaf strips.
[1]5 hours: On shaker, [2]15 hours: Stationary

3.2 Effect of cellulases on protoplast isolation from *in vitro* tissues

The protoplast yield from seedling shoots of *P.kawakamii* was greater from 2 % Seravac cellulase than any other concentration, either higher or lower (Table 8.4). The yield of protoplasts was greatly improved when the tissue was preplasmolyzed in mannitol before enzyme treatment. This is in agreement with the results of Yang *et al.* (1989). Preplasmolysis of tissue reduced the uptake into the cytoplasm of exogenously supplied enzymes, leakage of electrolyte and the osmotic shock during protoplast isolation (Cocking, 1972; Reusink, 1980).

Protoplasts were released more readily from mesophyll cells when tissue was incubated with Onozuka cellulase as compared to Seravac cellulase (Table 8.5). Protoplasts were released more readily from mesophyll cells of *P.tomentosa* than from *P.taiwaniana*.

This may be related to the difference in the leaf structure of the two species. Leaves of *in vitro* grown plantlets of *P.tomentosa* grow rapidly and cells are loosely structured. Mesophyll cells separated from the epidermis during incubation and thus resulted in better contact between enzyme and cell (Fig. 8.1). This explains the higher yield of protoplasts from leaf explant as compared to shoot explants. Protoplast yield from leaf explants was improved when the cultures were shaken during incubation (Table 8.6). Isolation of protoplasts by incubating tissues with a combination of pectinase and cellulase was superior to treatment with either of enzymes separately (Tables 8.3 and 6).

3.3 Effect of mannitol concentration on plasmolysis

The viability of *P.taiwaniana* cells was reduced when they were placed in a solution of mannitol (Table 8.7). Increasing concentrations especially above 0.7 to 0.9 M and the time of exposure to mannitol both affected cell viability negatively. The optimum concentration of mannitol for plasmolysis of suspension cells is between 0.5 to 0.6 M. Microscopic observation of plasmolysis revealed that the plasma membranes are strongly bound to the cell walls (Fig. 8.2-a) thus preventing normal and complete plasmolysis of many cells. This phenomenum was reported for *Betula* and *Glycine* cells and also affected the yield and viability of the protoplasts (Lee-stadelmann *et al.*, 1985; Smith *et al.*, 1989)

3.4 Effect of cellulases on isolation of suspension cells

Forty four percent of the cells in suspension treated with Seravac cellulase released viable protoplasts as compared to a yield of only 21 % when Onozuka R10 cellulase was used (Table 8.8). Onozuka R10 cellulase was effective in protoplast isolation of

mesophyll cells while Seravac cellulase was effective in protoplast isolation of suspension cells (Tables 8.5 and 8.8). This may be due to the differences in cell wall composition or variation in the activity of the brand of cellulase as reported by Fitzsimons and Weyers (1985).

3.5 Protoplast culture

Protoplasts derived from mesophyll cells of *P.tomentosa* and cultured on different combinations of NAA and BA or 2,4-D and BA failed to divide. The chromosomes of protoplasts derived from suspension cells of *P.taiwaniana* and cultured on DK_1NB_2 medium became dense after 5 days in culture and the first signs of division were visible after 7 days in culture (Fig. 8.2-b and c). However, division did not progress beyond the initial stages. The culture requirement for *Paulownia* protoplasts is still poorly understood and further research is required.

Table 8.4. Effect of Seravac cellulase concentration and preplasmolysis on the protoplast release from seedling shoots of *P.kawakamii*

Cellulase concentration	Number of protoplasts x 10^5 per g of seedling shoots	
	Direct enzyme treatment	Preplasmolysis prior to enzyme treatment
1.0 %	1.7	12
2.0 %	7.0	33
3.0 %	4.9	9

Table 8.5. Effect of cellulase brand on protoplast yield from mesophyll cells of *P.tomentosa* and *P.taiwaniana*

Cellulases	10^7 protoplasts per g of leaves	
	P.tomentosa	*P.taiwaniana*
Seravac	0.9	0.1
Onozuka R10	2.4	0.2

Table 8.6. Release of protoplast from mesophyll cells of *P.tomentosa* as affected by shaking the culture during incubation.

10^7 protoplasts per g of leaves	
Shaking	Stationary
2.2	0.7

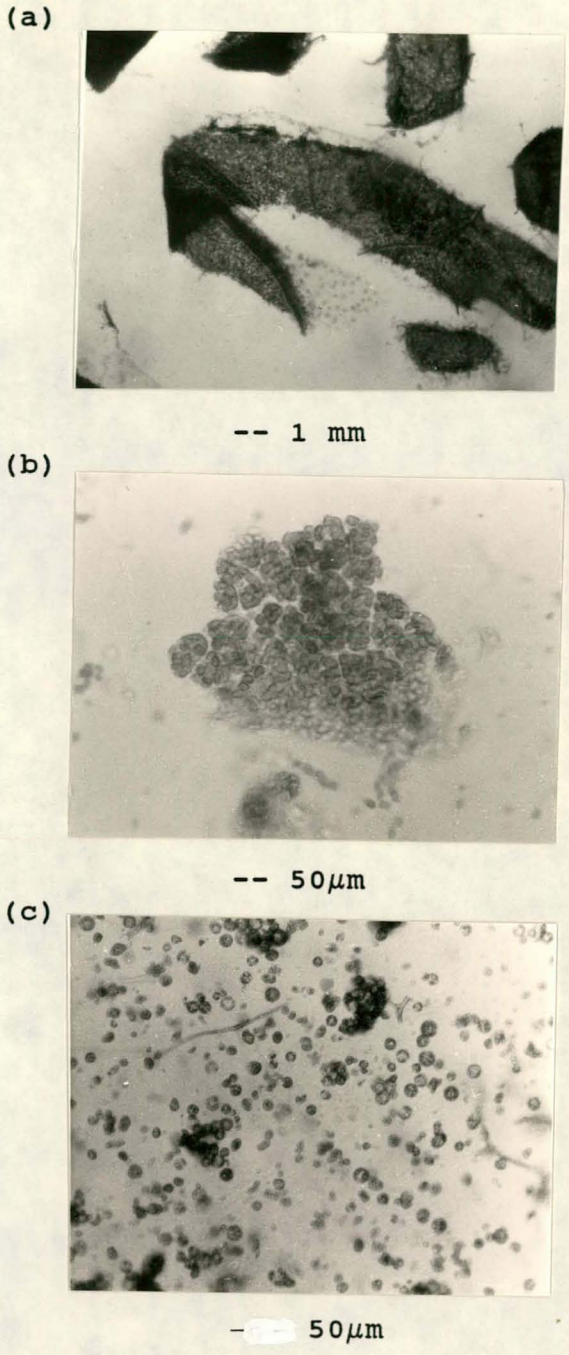


Fig. 8.1. Protoplast isolation: (a) epidermis separated from mesophyll cells of *P.tomentosa* leaf strips in culture; (b) mesophyll cells exposed; (c) protoplasts released from mesophyll cells of *P.tomentosa*

Table 8.7. Effect of mannitol concentrations on viability of suspension cells of *P.taiwaniana*

Culture period (hour)	Viability % in different Molarities of mannitol					
	0	0.5	0.6	0.7	0.8	0.9
1	84	79	80	68	67	60
3	84	75	75	68	55	43
5	83	70	72	60	50	40
15	80	52	48	49	36	35

Table 8.8. Effect of cellulase brand on protoplast release from suspension cells of *P.taiwaniana*

Cellulases	Cell density (10^7 ml^{-1})	Protoplast density (10^7 ml^{-1})	protoplast yield %
Seravac	28	6	21
Onozuka R10	34	15	44

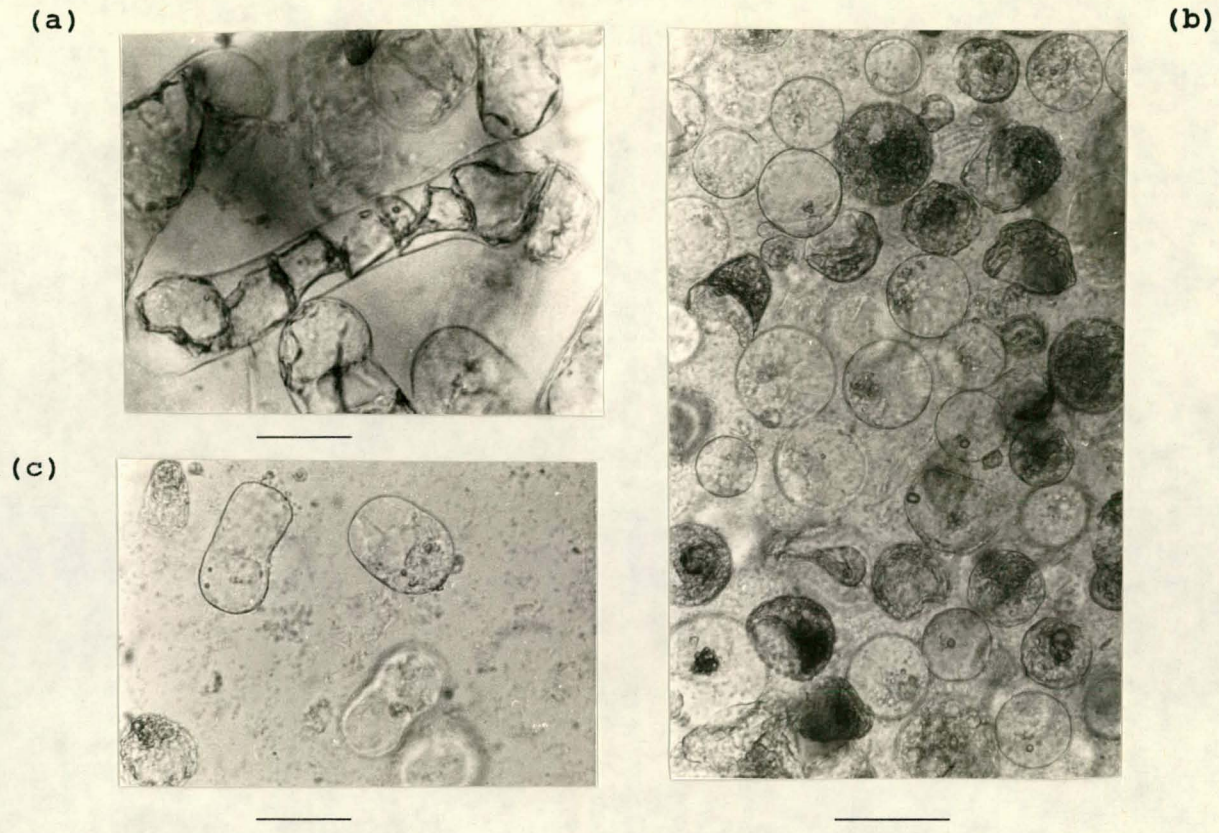


Fig. 8.2. Isolation and culture of protoplasts derived from suspension cells of *P.taiwaniana*: (a) plasmolysis of cells in 0.7 M mannitol; (b) hanging drop culture of protoplast, dense chromosomes were visible in some protoplasts; (c) initial stage of protoplast division. Bars represent 50 μm

4. REFERENCES

Attree, S.M., Bekkaoui, F., Dunstan, J.I. and Fowke, L.C., 1987. Regeneration of somatic embryos from protoplasts isolated from an embryogenic suspension culture of white spruce (*Picea glauca*). Plant Cell Rep. 6:480-483

Chand, P.K., Ochatt, S.J., Rech, E.L., Powers, J.B. and Davey, M.R., 1988. Electroporation stimulates plant regeneration from protoplasts of the wood medicinal species *Solanum dulcamara* L. J. Exp. Bot. 39:1267-1274

Cocking, E.C., 1972. Plant cell protoplasts - isolation and development. Ann. Rev. Plant Physiol. 23:29-50

Dunstan, D.I. and Thorpe, T.A., 1986. Regeneration in forest trees. In: Vasil, I.K. (Ed.) Cell culture and somatic cell genetics of plants Vol.3: Plant regeneration and genetic variability (pp. 221-241) Academic Press Inc., USA

Durzan, D.J., 1988, Application of cell and tissue culture in tree improvement. In: Ciba Foundation (Ed.) Applications of plant cell and tissue culture. (pp. 49-58) John Wiley & Sons Ltd., UK

Evans, D.A. and Bravo, J.E., 1983. Protoplast isolation and culture. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.) Handbook of plant cell culture Vol.1: Techniques for propagation and breeding. (pp. 124-176) Macmilllan Publishers Co., New York

Fitzsimons, P.J. and Weyers, J.D.B., 1985. Properties of some enzymes used for protoplast isolation. In: Pilet, P.E. (Ed.) The physiological properties of plant protoplasts. (pp. 12-23) Springer-Verlag, Berlin

Gupta, P.K. and Durzan, D.J., 1987. Somatic embryos from protoplasts of loblolly pine proembryonal cells. Bio/Tech. 5:710-712

Kitamura, Y., Morikawa, T. and Miura, H., 1989. Isolation and culture of protoplasts from cell suspension cultures of *Duboisia myoporoides* with subsequent plant regeneration. Plant Sci. 60:245-250

Lee-Stadelmann, O., Chung, I. and Stadelmann, E., 1985. Plasmolysis of *Glycine max* mesophyll cells: The use of octylguanadine and its implications in protoplast isolation. Plant Sci. 38:1-7

Ochatt, S.J., 1990. Plant regeneration from root callus protoplasts of sour cherry (*Prunus cerasus* L.). Plant Cell Rep. 9:268-271

Ochatt, S.J. and Caso, O.H., 1986. Shoot regeneration from leaf mesophyll protoplasts of wild pear (*Pyrus communis* var. *pyraster* L.). J. Plant Physiol. 122:243-249

Ochatt, S.J. and Power, J.B., 1988. An alternative approach to plant regeneration from protoplasts of sour cherry (*Prunus cerasus* L.). Plant Sci. 56:75-79

Ochatt, S.J., Cocking, E.C. and Power, J.B., 1987. Isolation, culture and plant regeneration of Colt cherry (*Prunus avium* x *pseudoserasus*) protoplasts. Plant Sci. 50:139-143

Ochatt, S.J., Escandon, A.S. and Martirez, A.J., 1989. Isolation, culture and plant regeneration of protoplasts of two shrubby *Oxalis* species from South America. J. Exp. Bot. 40:493-496

Oka, S. and Ohyama, K., 1985. Plant regeneration from leaf mesophyll protoplasts of *Broussonetia kazinoki* Sieb. J. Plant Physiol. 119:455-460

Park, Y.G. and Son, S.H., 1992. *In vitro* shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (*Populus nigra* x *P.maximowiczii*). Plant Cell Rep. 11:2-6

Patat-Ochatt, E.M., Ochatt, S.J. and Power, J.B., 1988. Plant regeneration from protoplasts of apple rootstocks and scion varieties (*Malus* x *domestica* Borkh.). J. Plant Physiol. 133:460-465

Potrykus, I., Harms, C.T. and Lorz, H., 1979. Multiple-drop-array (MDR) technique for the large-scale testing of culture media variations in hanging microdrop cultures of single cell systems I: The technique. Plant Sci. Lett. 14:231-235

Rao, P.S. and Ozias-Akins, P., 1985. Plant regeneration through somatic embryogenesis in protoplast cultures of sandalwood (*Santalum album* L.). Protoplasma 124:80-86

Reusink, A.W., 1980. Protoplast of plant cells. Methods Enzymology 69:69-84

Russell, J.A. and McCown, B.H., 1988. Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Rep. 7:39-62

Saito, A., 1980a. Isolation of protoplasts from mesophyll cells of *Paulownia* and *Populus*. Bull. For. & For. Prod. Res. Inst. 309:1-6

Saito, A., 1980b. Fusion of protoplasts isolated from somatic cells of tree species. Bull. For. & For. Prod. Res. Inst. 309:7-14

Schöpke, C., Müller, L.E. and Kohlenbach, H-W., 1987. Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex Fr.). Plant Cell, Tissue & Organ Cult. 8:243-248

Smith, M.A.L., Palta, J.P., McCown, B.H. and Steffen, K.L., 1989. Plasmolytic behavior of the donor cell may affect protoplast response. Physiol. Plant. 76:201-204

Sticklen, M.B., Domir, S.C. and Lineberger, R.D., 1986. Shoot regeneration from protoplasts of *Ulmus* x 'Pioneer'. Plant Sci. 47:29-34

Tsai, C.K., 1988. Plant regeneration from leaf callus protoplasts of *Actinidia chinensis* var. *chinensis*. Plant Sci. 54:231-235

Vardi, A., Spiegel-Roy, P. and Galun, E., 1982. Plant regeneration from *Citrus* protoplasts: Variability in methodological requirements among cultivars and species. Theor. Appl. Genet. 62:171-176

Yang, J.C., Ho, C.K., Chang, S.H. and Huang, F.H., 1989. The isolation of mesophyll protoplasts in *Paulownia taiwaniana*. In: Yang, J.C., Lin, T.P. and

Wang, W.Y. (Eds.) Sino-American Workshops on Forest Biotechnology and Molecular Biology. (pp. 51-60) Nat. Sci. Council, Rep. of China

CHAPTER 9

SUMMARY AND CONCLUSION

Paulownia species are fast growing trees with high economic value for furniture, toys, plywood, crates and musical instruments. They have been introduced to five continents as ornamental trees or for wood production. Witches' broom caused by a mycoplasma-like organism has damaged the economic value of the genus in eastern Asia. Plant cell and tissue culture have proved useful tools for tree improvement in providing disease free material, preserving germplasm, broadening the gene pool for desired trait selection and creating a novel tree by gene transformation.

The aim of this study was to establish an *in vitro* system for organogenesis, embryogenesis, tissue and callus culture, cell suspension and regeneration, and protoplast isolation and culture for the genus *Paulownia*. Four species: *P.fortunei*, *P.kawakamii*, *P.tomentosa* and *P.taiwaniana* which represent the 3 sections of the *Paulownia* genus were used.

An extensive review of literature was made and the application of plant cell and tissue culture on conventional tree breeding discussed. Many reports on organogenesis of *Paulownia* species exist, there are, however, only a few reports on embryogenesis, callus culture and anther culture and no reports on cell suspension or protoplast culture could be found. None of the reviewed articles attempted to compare the organogenetic potential of *in vitro* culture for the genus *Paulownia* .

To obtain *in vitro* seedlings for experimental usage germination of the seeds of four *Paulownia* species sown in different growing media was studied. Germination of both mature and immature seeds for all *Paulownia* species was stimulated by soaking seeds in a 3.5 % sodium hypochlorite solution. Pretreatment of the seeds with 70 % alcohol for 1 min before soaking in sodium hypochlorite caused higher germination percentages than sodium hypochlorite treatment alone. MS medium inhibited germination of mature seeds, but germination of immature seeds was not affected to the same extent. Analyses of the effect of individual components of MS medium on the germination of *P.tomentosa* revealed that sucrose, total salts and some macronutrients were the major inhibitory factors. *Paulownia* seeds should therefore be germinated on water solidified with 0.6 % agar before transfer to MS medium for *in vitro* growth.

Organogenesis of various explants of four *Paulownia* species was studied. Six explant types: cotyledon, hypocotyl, shoot tip, nodal, internodal and leaf explants were tested. Explants were cultured on MS medium supplemented with different combinations of NAA and BA. Histological study revealed that adventitious bud formation originated from the callus tissue (indirect organogenesis) or from the subepidermal parenchyma cells without a callus interphase (direct organogenesis). All explants of *P.tomentosa* and *P.taiwaniana* and cotyledon and hypocotyl explants of *P.fortunei* and *P.kawakamii* formed adventitious buds within the concentration range of 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA. Hypocotyl explants produced more adventitious buds than the other explants, while leaf explants produced the least. Adventitious bud and root formation on the other four explants for *P.fortunei* and *P.kawakamii* occurred over a wide range of NAA + BA combinations. Adventitious bud formation of internodal explants of *P.tomentosa* was inhibited by Biolab agar but not by Difco agar. Biolab agar induced more callus growth than Difco agar for all four species. *P.fortunei* and *P.tomentosa* formed callus

more readily than the other two species. It appears that the organogenesis patterns and callus induction are different not only among species but also among explant types.

Extensive vitrification of *in vitro* shoot subcultures occurred on gelrite medium without hormones. Where hormones were added on media solidified with either agar or gelrite, vitrified adventitious buds formed from explant cultures. Vitrification of adventitious buds induced from internodal explants of the four *Paulownia* species was studied. Different concentrations of agar, cobalt, inositol, MS macrosalts, growth regulators and different light intensities failed to eliminate vitrification effectively. However, increasing light intensities increased adventitious bud formation. *P.taiwaniana* formed fewer vitrified shoots than *P.fortunei* and *P.kawakamii*. Gelrite formed more adventitious buds but caused more vitrification than agar. The morphological characteristics and growth of normal and vitrified shoots were studied when subcultured on different gelling agents and concentrations. Vitrified shoots of *P.fortunei* and *P.tomentosa* produced more callus on the cut surfaces of shoots whereas the other two species formed more axillary shoots. Vitrified shoots had a higher growth rate than normal shoots but the ability to form roots did not differ from normal shoots. Increasing the concentration of gelling agents inhibited shoot growth except for *P.kawakamii* which were equally and inherently slow on all concentrations of all gelling agents tested. Vitrified shoots decreased water content and reverted to normal shoots when cultured on 0.8% or higher Difco agar, but retained vitrification on gelrite and mixtures of gelrite and agar. This suggests that it is not necessary to reduced vitrified shoot formation on shoot formation medium as it is always companied by the unfavorable suppression of adventitious bud formation. Healthy *in vitro* plantlets could be obtained from either vitrified or normal shoots by rooting on MS medium solidified with 0.8 % Difco agar.

Induction of somatic embryogenesis in four species of *Paulownia* was investigated. Ovules of *P.fortunei* at the globular proembryo stage cultured on Radojevic (1979) , MSG (Amerson *et al.*, 1988) and DCR (Gupta & Durzan, 1986) media supplemented with auxins and cytokinins developed into mature embryos or formed callus. However, both callus and embryos failed to develop further when subcultured. Mature and immature seeds cultured on various media germinated in low numbers. Germinating embryos were cultured on Radojevic medium (1979) or MGM medium (Muralidharan *et al.*, 1989) supplemented with 2, 5 % sucrose and picloram, 2,4-D or NAA combined with kinetin or BA in light or dark. None of these treatments succeeded in forming embryogenic tissue. Embryogenesis could not be induced in *P.tomentosa* by the procedure described by Radojevic (1979). The key factors are thought to be differences in embryo stage and the genotype used both of which require further research.

The behavior of callus tissue derived from different explant types of the four *Paulownia* species cultured on MS medium plus various combinations of NAA + BA or 2,4-D + kinetin was studied in MS media containing NAA or 2,4-D plus BA or 2,4-D plus kinetin. Organogenetic calli compact in structure and greenish white in colour were induced in low levels of NAA (< 0.5 mg l^{-1}) or in combinations of NAA and BA or in low levels of 2,4-D plus kinetin. Friable calli white or grey in colour developed in high 2,4-D levels (0.1 mg l^{-1}) plus kinetin. Subcultures of organogenetic calli derived from a medium (0.5 mg l^{-1} NAA and 5 mg l^{-1} BA) which induced adventitious bud formation could not be maintained when subcultured. *P.taiwaniana* organogenetic callus developed on a medium containing 0.1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin formed few adventitious buds but formed many adventitious buds when subcultured on 0.01 mg l^{-1} NAA and 10 mg l^{-1} BA. *P.kawakamii* did not respond in this way. Most calli whether organogenetic or friable turned brown when subcultured. Treatments

with antioxidant did not reduce browning. Subcultures of callus tissues with adventitious buds reduced degree of browning as compared to subcultures without adventitious buds.

Cell suspensions of *P.tomentosa* were obtained from friable calli which were obtained by culturing shoot tip explants on MS medium containing 2.5 mg l^{-1} NAA and BA. The cell suspensions of remaining three species turned brown and failed before the 3rd subculture. Suspension cell growth of *P.tomentosa* was exceptionally slow making it difficult to quantify the cell growth cycle. Cell suspensions of *P.tomentosa* and *P.taiwaniana* were also obtained without an interphase of callus by culturing seedling shoots on MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. Seedling shoots of *P.fortunei* and *P.kawakamii* treated the same way turned brown and died before the 3rd subculture. Cultures were kept in the dark on a gyratory shaker at 100 rpm. Cell densities of 4.5 x 10^5 were achieved by using 200 mg explants. Most cells (75 - 95 %) were viable and cell suspensions of *P.tomentosa* and *P.taiwaniana* had a rapid growth rate. The cultures were maintained for more than one year by regular subculturing. The same medium was used to subculture *P.tomentosa* cells, but for successful subcultures of *P.taiwaniana* 2,4-D and kinetin concentration was decreased and the addition of NAA and BA was necessary. Suspension cells of *P.tomentosa* were large and spherical in shape while those of *P.taiwaniana* were either elongated or small and spherical. Stable growth cycles for both species were determined by various parameters such as pH, settled cell volume, packed cell volume, optical density, fresh and dry weights. Fresh and dry weights were less variable and more accurate than the other parameters. This method of establishing cell suspension cultures is effective and time-saving compared to the generally accepted method of using callus tissue. The production of friable callus on agar suitable for suspension culture is eliminated. Callus formation from suspension cells for both species was

achieved by cell plating or transferring suspension cells into liquid MS medium containing NAA and BA. Organogenesis from callus was, however, not achieved.

Isolation of protoplasts from *in vitro* explants of *P.tomentosa*, *P.kawakamii* and *P.taiwaniana* and suspension cells of *P.taiwaniana* was studied. Leaves of *P.tomentosa* treated with Seravac pectinase yielded more free mesophyll cells than other brands of pectinase. High yields of protoplasts were obtained when explants were incubated in enzyme solutions consisting of 0.6 % pectinase, 0.6 % hemicellulase and 2 % Onozuka R10 cellulase. High yields of protoplasts from suspension cells of *P.taiwaniana* were obtained when cells were treated with 2 % Seravac cellulase. Suspension cells of *P.taiwaniana* exhibited strong membrane-to-cell wall attachment and many cells failed to plasmolyze completely in a mannitol solution. Injury to the cells was evident in concentrations in excess of 0.6 M mannitol and incubation times longer than 5 hours. Protoplasts from mesophyll cells of *P.tomentosa* failed to divide while initial stages of division in protoplasts from suspension cells of *P.taiwaniana* were evident but division was not completed.

The following conclusions are drawn from this study:

(1) Germination of *Paulownia* is enhanced by sterilization (alcohol and NaOCl) and surfactant (alcohol and Tween 20).

(2) MS medium inhibited seed germination in all four species of *Paulownia* tested but especially in *P.fortunei* and *P.taiwaniana*. To obtain contaminant-free seedling for experimental purposes seeds should be germinated on agar and young seedlings plants transferred to MS medium.

(3) Organogenetic potential and callus growth differed between either species or explant types of the same species when cultured on various combinations of NAA and BA. However, adventitious bud formation was optimized when explants of all four species were cultured on MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA and solidified with 0.8 % Difco agar.

(4) Gelling agents affected callus growth for all four species and adventitious bud formation of *P.tomentosa*.

(5) A histological study revealed that adventitious buds originate from callus or from subepidermal parenchyma cells.

(6) Vitrification occurred in *in vitro* shoot subcultures on MS medium solidified with gelrite and adventitious buds formed from explants cultured on medium containing hormones. Vitirified shoots, however, could be reverted to healthy plantlets by transferring them on 0.8 % or higher Difco agar.

(7) The ability to form adventitious buds decreased with explant age and could not be maintained in subcultured callus, except for *P.taiwaniana* callus derived from 2,4-D and kinetin medium which formed many adventitious buds when subcultured on NAA and BA medium.

(8) Browning of callus subcultures was reduced when callus contained adventitious buds.

(9) Zygotic embryo development or callus growth were induced from ovules at the globular proembryo stage of *P.fortunei*. Embryos cultured in different basal

medium, auxins and cytokinins and sucrose concentration failed to form embryogenic callus. The relationship between the ovule development stage or the effect of different genotypes on somatic embryogenesis require further study.

(10) Cell suspensions of *P.tomentosa* and *P.taiwaniana* without the interphase of friable callus were established. Cell growth cycles for both species were determined by several parameters and have been maintained by regular subculturing for more than one year. Cell plating and cell aggregate cultures formed callus but failed to regenerate.

(11) A method of protoplast isolation from *in vitro* shoots and suspension cells is reported. Protoplasts from mesophyll cells of *P.tomentosa* failed to divide while initial stages of division in protoplasts derived from suspension cells of *P.taiwaniana* was observed.